University of Alberta

The Role of MUC1 in Vascular Adhesion: An Essential Step in Breast Cancer Metastasis

by

Lyle Henry John Regimbald

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Experimental Pathology

Department of Laboratory Medicine and Pathology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, *The Role of MUC1 in Vascular Adhesion: An Essential Step in Breast Cancer Metastasis,* submitted by Lyle Henry John Regimbald in partial fulfillment of the requirements for the degree of Master of Science in Experimental Pathology

Judith Hugh

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Dr. Robert Stinson

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Date

July 5/96

Dedication

This thesis is dedicated to the memory of my mother who is always an inspiration to me, and whose teachings and support are invaluable.

Abstract:

The MUC1 mucin is expressed on normal breast epithelium, and in a uniquely underglycosylated form in ninety percent of breast cancers. MUC1 carries sialylated carbohydrate ligands for endothelial selectins. Hence, it is possibly involved in the initial weak adhesion or "rolling" of tumor cells on vascular endothelium during metastatic spread. This thesis demonstrates that MUC1 binds to intercellular adhesion molecule-1 (ICAM-1); a molecule involved in the final or "firm attachment and transendothelial migration" phases of vascular adhesion. Monoclonal antibodies against ICAM-1 and against MUC1 inhibited adhesion of human and transfected mouse MUC1 positive cell lines to human umbilical vein endothelial cell (HUVEC) monolayers, to ICAM-1 transfected cells, and to immobilized recombinant human ICAM-1—Ig fusion protein (rhICAM-1). Purified soluble MUC1 pretreatment of rhICAM-1 was also an effective inhibitor of adhesion using the rhICAM-1 adhesion system. MUC1 may thus be a multi-functional adhesion molecule capable of mediating more than one phase of tumor cell—endothelial cell adhesion. As such, MUC1 may be critical to the process of blood borne metastases in breast cancer.

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List of Abbreviations

1. ICAM-1	Intercellular adhesion molecule-one
2. rhICAM	Human recombinant intercellular adhesion molecule-one—Immunoglobulin G fusion protein
3. HUVEC	Human umbilical vein endothelial cells
4. MAdCAM-1	Mucosal addressin cell adhesion molecule-one
5. ECM	Extracellular matrix
6. MTC	Metastatic tumor cells
7. MLC	Migrating leukocytes
8. E selectin	Endothelial selectin
9. P selectin	Platelet selectin
10. $sLe^{x/a}$	Sialylated Lewis x & a antigens
11. Ig	Immunoglobulin
12. LAD	Leukocyte adhesion deficiency
13. VCAM-1	Vascular cell adhesion molecule-one
14. PECAM-1	Platelet endothelial cell adhesion molecule-one
15. EGF	Epidermal growth factor
16. IL-1	Interleukin-one
17. LFA-1	Lymphocyte function-associated antigen-one
18. Mac-1	CD11b/CD18 β2 integrin
19. Ab	Antibody
20. mAb	Monoclonal antibody
21. kD	Kilo-Dalton

22.	p150,95	protein 150,95
23.	TNF	Tumor necrosis factor
24.	IL-4	Interleukin-four
25.	GlyCAM-1	Glycosylation dependent cell adhesin molecule-one
26.	PSGL-1	P selectin glycoprotein ligand-one
27.	VNTR	Variable number of tandem repeats
28.	sTn	Sialylated Tn antigen
29.	TF	TF antigen
30.	RPMI	RPMI 1640 growth medium
31.	FCS	Fetal calf serum
32.	PBS	Phosphate buffered saline
33.	PE	Phycoerythrin
34.	DMEM	Dulbecco's modified Eagle's medium
35.	rpm	revolutions per minute
36.	NP-40	Nonidet P-40
37.	BSA	Bovine serum albumin
38.	HCL	Hydrochloric acid

Chapter 1

INTRODUCTION

1.1. Background

Cancer is a collective term for approximately 200 distinct clinicopathological conditions related by the properties of uncontrolled cell growth and metastatic spread of these abnormal cells.

Breast cancer will affect one in nine Canadian women, a rate among the highest in the world with those in Alberta slightly higher than the national average (1). It is the primary cause of premature death of women in Canada and accounts for the loss of 95,000 potential years of life annually among Canadian women (1).

Metastases are responsible for the majority of treatment failures and deaths in cancer patients. This is particularly important in breast cancer which is often a systemic disease at the time of diagnosis. Therefore intensive ongoing research aimed at understanding and controlling metastatic spread, is a central issue in the treatment of breast cancer.

Metastases can be described as the dissemination of malignant cells throughout the body and their survival to form secondary growths (2). This constitutes a highly complex process dependent on many host and tumor properties. Acquisition of metastatic potential results from the random accumulation of several specific genetic and cellular changes.

1.2. The Metastatic Process

Successful metastatic spread requires that a tumor cell(s) is able to dissociate from the primary mass, attach to, degrade and penetrate the extracellular matrix (ECM), and / or the basement membrane, enter the circulation, home to a secondary site, leave the circulation, and direct angiogenesis (Figure 1).

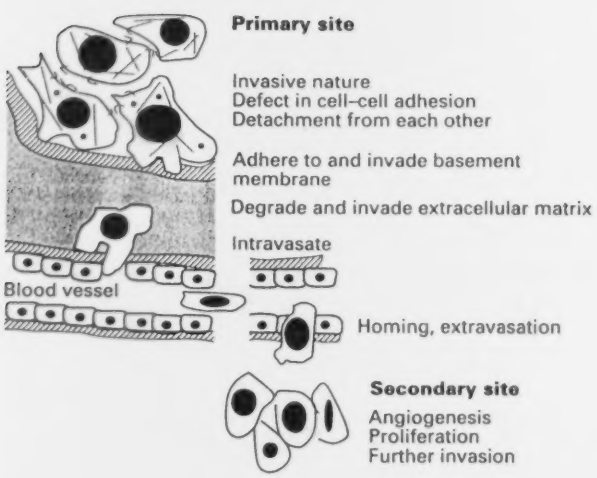


Figure 1. Metastatic process of cancer cells. Invasion of the basement membrane and extracellular matrix, intravasation, "homing" and extravasation are indicated. (taken from ref. 2)

1.3. Tumor cell - Vascular Endothelial cell Adhesion and its similarity to Normal Inflammatory cell - Endothelial cell Adhesion

Entrance into the circulation presents a very hostile environment to invading tumor cells which must overcome high velocity collisions and immune surveillance. A small fraction of these blood borne tumor cells survive to arrest in the microvasculature of secondary target organs. The adhesive interactions between tumor cells and target vascular endothelium are an essential and highly specialized component of the metastatic cascade and represent the major topic of this thesis. Over the last ten years it has been shown that metastatic tumor cells (MTC) adhere to the endothelium using mechanisms similar to those employed by normal extravasating (migrating) leukocytes (MLC) (3 - 5). These adhesive interactions must overcome circulatory shear forces and are dependent on multiple pairings of "lock and key" type interactions between adhesion molecules on the surfaces of MLC and endothelial cells (6) (Figure 2).

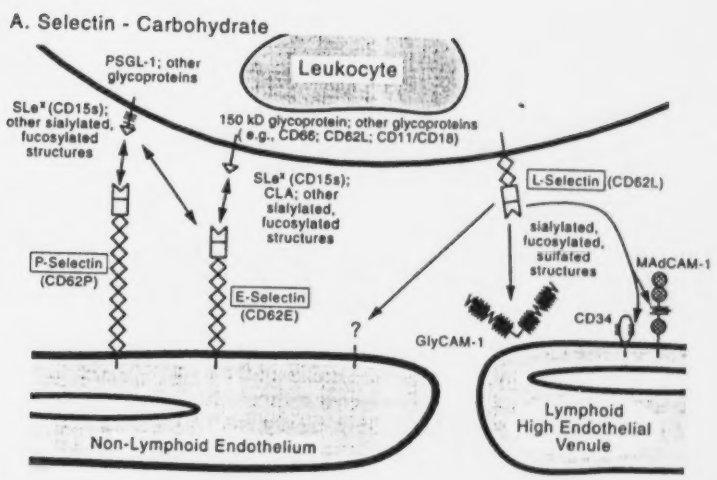


Figure 2. Adhesion receptor - ligand pairing displayed as carbohydrate binding to selectin structures (taken from ref. 41)

1.3.1. The Vessel Wall

Lining the interior of blood vessels is a single layer of endothelial cells which encircle the vessel lumen. These endothelial cells express a variable repertoire of surface proteins (adhesion molecules) which selectively participate in adhesion of MLC (and MTC) by binding surface proteins on MLC. These endothelial adhesion molecules are transiently induced by exposure to inflammatory cytokines, bacterial lipopolysaccarhide, and other agents.

The endothelial cells produce a thin, continuous, fibrous basement membrane located on the abluminal surface of the monolayer. The subendothelial basement membrane consists of a tightly regulated composition which includes, von Willebrand factor, fibronectin, thrombospondin, laminin, nidogen,proteoglycans, and collagen types III, IV, V, and VIII (7). The matrix organization and composition of the basement membrane varies depending on cytokine induced endothelial cell stimulation (7).

1.3.2. MLC - Endothelial Cell Adhesion Cascade

An ordered sequence of three adhesion events with increasing avidity has been observed in MLC extravasation, (Figure 3) using intravital microscopy (8). These studies show that MLC initially "roll" along the endothelium of post capillary venules adjacent to sites of inflammation. Rolling is a necessary prerequisite to the second or "firm adhesion" phase (9). The final or "transmigration" phase involves the exit of MLC from the vessel by protruding and squeezing between endothelial cells into subendothelial tissue.

1.3.3. Implicated Adhesion Molecules

Many of the adhesion molecules which mediate these interactions have been elucidated through both *in vitro* and *in vivo* studies (10 - 14). *In vitro* studies examining leukocyte adhesion to endothelial selectins have shown that neutrophils roll on lipid bilayers containing E selectin (15), and P selectin (16). Consistent with these findings, antibodies to E selectin effectively inhibited neutrophil accumulation in the inflammed peritoneum and lungs of rats (17). The corresponding ligands on MLC which bind the endothelial selectins are sialylated or fucosylated carbohydrates such as the sialylated Lewis antigens (sLe^x, & sLe^a) (3 - 5, 18, 19). *In vivo* studies of leukocyte adhesion deficiency (LAD) type II neutrophils which lack sLe^x (E selectin ligand) (20) failed to demonstrate efficient rolling of these neutrophils on E selectin expressing venule endothelium, unlike normal neutrophils which did roll and subsequently firmly adhere (21).

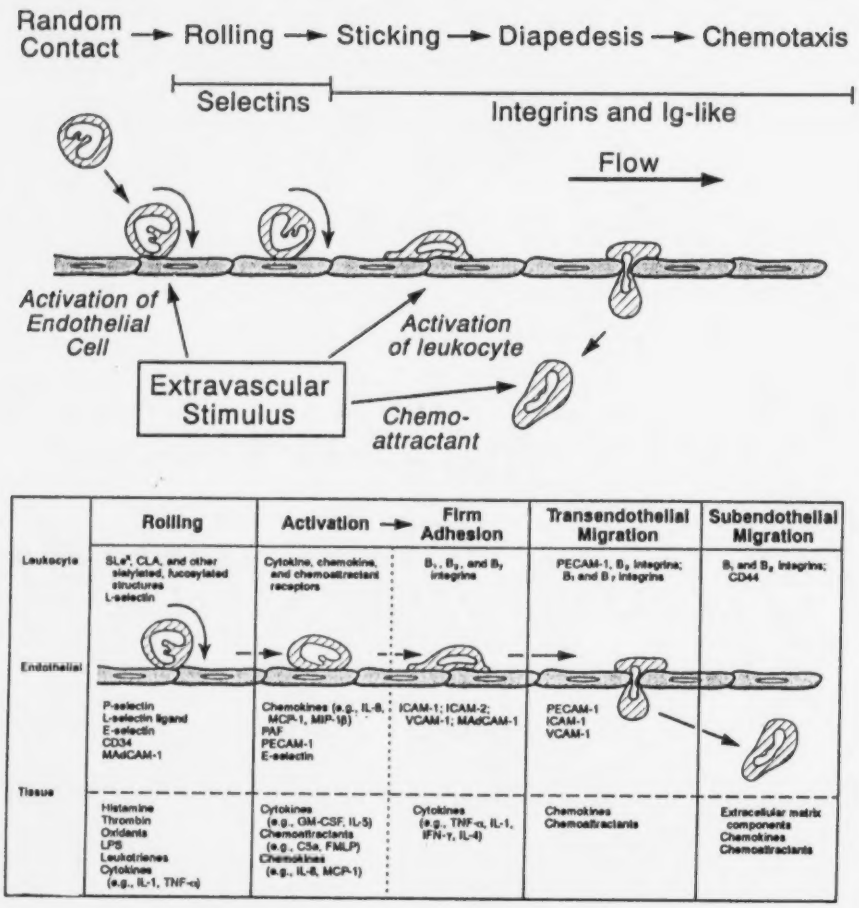


Figure 3. The leukocyte - endothelial cell adhesion cascade consists of a "rolling" phase followed by firm attachment and extravasation. Each phase is mediated by distinct and specialized adhesion receptor - ligand pairings. (taken from ref. 41)

Firm attachment to the endothelium occurs following the initial rolling or tethering of circulating cells. This phase is mediated by high affinity adhesion between MLC surface β_1 & β_2 integrins and endothelial immunoglobulin (Ig) superfamily molecules such as ICAM-1, ICAM-2, and vascular cell adhesion molecule-1 (VCAM-1). Intravital microscopy studies using neutrophils from LAD type I patients which express little or no functional β_2 integrins (22) exhibited normal rolling but no firm adhesion or transendothelial migration upon chemotactic stimulation (21). The high affinity interactions between Ig superfamily molecules and integrins are thought to be activated by chemoattractants, and by ligation of the selectins (23). This activation is a rapid process and results in de novo synthesis and increased expression of endothelial ICAM-1, VCAM-1 and others (24), and in increased avidity caused by conformational changes in the integrin heterodimers (25).

The final phase of the MLC - endothelial cell adhesion cascade involves migration of arrested cells over the endothelium to points of endothelial intercellular junctions, and subsequent diapedesis between adjacent endothelial cells to enter extravascular tissue. This ordered and complex sequence of events requires reversible adhesion, as seen by cyclic modulation of integrin receptor avidity (26), cytoskeletal modification to propel the cell forward, and release of proteolytic enzymes to tunnel its way into and through the ECM (2).

The adhesion molecules involved in traversing the endothelium, are likey the same as those utilized in the firm attachment phase. Using in vitro transendothelial migration studies, neutrophils from LAD type I patients, or normal neutrophils treated with blocking antibody to CD18 (β_2 subunit) were unable to transmigrate in response to chemotactic stimuli (27). Similar studies

showed that endothelial ICAM-1 was implicated (28), coinciding with β_2 integrin involvement. In 1993, another endothelial Ig superfamily molecule, PECAM-1 (CD31) was shown to be involved in transendothelial migration (29).

1.3.4. Endothelial Adhesion Molecules

E selectin is the major selectin expressed by endothelial cells. It shows structural features common to all three selectins. These are the presence of an NH_2-terminal C-type (Ca^{2+} dependent) lectin-like binding domain, an epidermal growth factor (EGF)-like region, a variable number of consensus repeat sequences similar to those of complement regulatory proteins, a membrane spanning region, and a short cytoplasmic region (30). E selectin was originally implicated in the adhesion of neutrophils and several leukemic cell lines to endothelium which showed expression of this 115 kD antigen following stimulation with interleukin -1 (IL-1) (31). It contains a core protein of 64 kD with 11 potential N-glycosylation sites.

Mapping of E selectin domains has shown that the NH_2-terminal nine amino acids of the lectin domain and an epitope within the EGF-like region are important for ligand binding suggestive of carbohydrate ligands (32). In the early 1990's the fucosylated, tetrasaccharide, sLe^x was identified as a ligand for E selectin (33, 34). This and other fucosylated lactosamines are heavily expressed on neutrophils, monocytes (35), and natural killer cells (36).

ICAM-1, first identified in 1986 (37), is expressed on leukocytes, fibroblasts, epithelial cells, and endothelial cells and thus, is likely a crucial molecule in various types of cell—cell adhesive interactions. It is a heavily glycosylated, single chain molecule of the immunoglobulin superfamily. It has a core protein

of 55 kD with five extracellular immunoglobulin - like (Ig-like) domains (38). It has been suggested that this tandem domain duplication may be an evolutionary mechanism that creates multiple and independent binding sites for various adhesion moieties (39).

The cytoplasmic domain of ICAM-1 has been shown to be linked to the cytoskeleton through attachment to α actinin (40) which serves to anchor actin filaments to the cell membrane. As with other adhesion molecules this linkage to the cytoskeleton may localize ICAM-1 within specific regions of the endothelial cell membrane to facilitate circulating cell adherence and transmigration (41).

The first described ligands for ICAM-1 were the β_2 integrins LFA-1 and Mac-1. More recently, a non - integrin molecule, leukosialin (CD43) which is a heavily glycosylated sialomucin, was shown to bind ICAM-1(42). Whereas the binding site on ICAM-1 for CD43 has not been defined, LFA-1 and Mac-1 (β_2 integrins) bind specifically to the first and third Ig-like regions respectively. ICAM-1 mediated adhesion to LFA-1 requires the presence of divalent cations (43 - 45), is temperature dependent (46, 47), and requires a functional cytoskeleton (48).

ICAM-1 is involved in T cell mediated killing, T and B lymphocyte responses, natural killer cell function, Ab dependent cellular cytotoxicity, and adherence of leukocytes to endothelial cells, fibroblasts, and epithelial cells (49 - 51). In malignant melanoma (52 - 54) and gastric carcinoma (55), the development of metastatic potential has been correlated with tumor cell expression of ICAM-1.

VCAM-1 is another inducible Ig superfamily molecule found on the surface of stimulated endothelial cells. Cloning of VCAM-1 suggested seven extracellular

Ig like domains with a core protein of 81 kD containing seven potential N-glycosylation sites (56). Similar to ICAM-1, VCAM-1 contains two known ligand binding sites suggesting the possibility of multiple ligand recognition. These ligand binding sites have been localized to Ig domains one (57), and four (110). The integrins $\alpha_4\beta_1$ (59), and $\alpha_4\beta_7$ (60) have been described as ligands for VCAM-1.

Integrins are heterodimeric transmembrane proteins that bind to cytoskeletal proteins and transduce extracellular signals (61). There are eight subfamilies of integrins defined by the association of variable alpha chains with a common beta chain. Only three of these subfamilies have been clearly implicated in MLC adheson to endothelium: the β_2 integrins (LFA-1, Mac-1, & p150,95) (62), which bind ICAM-1 & ICAM-2, the β_1 integrin VLA-4 ($\alpha_4\beta_1$) (59), and the β_7 integrin $\alpha_4\beta_7$ (60) which bind VCAM-1.

1.3.5. Regulation of Adhesion Molecule Expression

Adhesive interactions between circulating cells and endothelial cells depend on the regulated expression of adhesion molecules on both cell types. This involves quantitative changes in surface expression and qualitative changes in avidity. Most evidence suggests that quantitative changes in endothelial adhesion molecules are most important while qualitative changes in circulating cell adhesion molecules predominate (41). The presence and quantity of three major endothelial adhesion molecules, E selectin, ICAM-1, & VCAM-1 are determined by cytokine (and other agonists) influences on transcriptional regulation of protein synthesis, and on the cellular expression kinetics (41).

Various combinations of cytokines or inflammatory mediators may lead to the differential induction of endothelial adhesion proteins. Some agonists are quite

selective for induction of only certain adhesion molecules while others induce several proteins (41). Specific combinations of cytokines may have both synergistic or antagonistic effects on adhesion molecule expression (63). For example, endothelial E selectin, ICAM-1, and VCAM-1 are induced by IL-1, and TNFα, while IFNγ induces ICAM-1 alone (41). The combination of TNFα and IL-4 is synergistic to VCAM-1 expression but opposes the normal enhancing effect of TNFα on E selectin expression (63). These phenomena allow for multiple levels of regulation, and precise modulation of endothelial adhesion molecule expression. This is likely important in the recruitment of specific leukocytes to sites of inflammation or metastatic spread.

Induction of three major endothelial adhesion molecules; E selectin, ICAM-1, and VCAM-1 show differing kinetics which is likely required for selective recruitment of blood borne cells. *In vitro* stimulation of endothelial cells shows peak surface expression of E selectin at 4 h post stimulation with a return to basal levels within 24 h (64). Surface expression of VCAM-1 and ICAM-1 peaks at 6 and 12 h respectively and both of these molecules persist for at least 72 h (65). These temporal differences in endothelial adhesion molecule expression at sites of inflammation likely account for the early recruitment of neutrophils followed later by the "chronic inflammatory cells" or lymphocytes.

1.3.6. MTC - Endothelial Cell Adhesion

A crucial step in the metastatic cascade is the arrest of MTC in the venous or capillary bed of the target organ. Similarities have been observed between MLC and MTC adhesion to vascular endothelium. Both MLC (41) and MTC (66) have been shown to adhere more effectively to endothelium activated with inflammatory cytokines. This enhanced adhesion was shown to result from

upregulation of endothelial adhesion molecules (67) recognized by both migrating cell types.

1.3.7. Endothelial Cell Adhesion Molecules important in Tumor Cell Adhesion

As in MLC rolling, MTC rolling has been demonstrated to depend on endothelial E selectin (68), and MTC have been shown to bind E selectin via their carbogydrate ligands (sLe^x or sLe^a) (4). VCAM-1 on cytokine activated endothelial cells binding to $\alpha_4\beta_1$, and $\alpha_4\beta_7$ integrins on the surface of migrating cells has been observed to support adhesion not only of MLC (41), but MTC as well (69). Similarly, another Ig superfamily member, PECAM-1 (CD31) has been shown to mediate adhesion of both MLC (41), and MTC (70) to endothelium.

Curiously, although ICAM-1 is an important endothelial adhesion molecule involved in MLC - endothelial cell adhesion it has not to been documented to mediate epithelial tumor cell - endothelial cell adhesion. This thesis presents evidence of a novel MTC ligand for endothelial ICAM-1; the mucin MUC1.

1.4. The Sialomucins

Since the early 1990's, investigators of circulating cell—endothelial cell adhesion have discivered a new family of adhesion molecules; the sialomucins. These include the endothelial mucins GlyCAM-1, CD34, and MAdCAM-1 and the leukocyte mucins PSGL-1 and CD43 (71). These molecules generally consist of a threadlike protein backbone containing several heavily glycosylated regions (Figure 4). The majority of the carbohydrate side chains are O-linked to the protein core, which confers important structural implications in mucins.

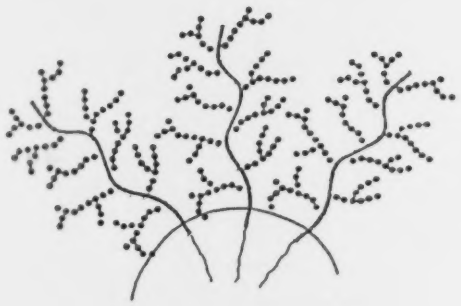
Figure 4. General Mucin structure. Extended protein backbone (—) containing a high density of O-linked glycans (|) and some N-linked glycans (Y). The N-terminus (N) and C-terminus (C) are represented.

The steric effect of these carbohydrates coupled with their high net negative charge, function to straighten the protein backbone making mucins long rigid molecules protruding far above (200 - 300 nm) the surrounding glycocalyx (72) (Figure 5). O-linkage also confers optimal exposure and high multiplicity of the terminal sugars (71) for interaction with appropriate counter-receptors (ie. selectins).

Other members of the mucin family are often found overexpressed in cancer cells. Increased production of mucin glycoproteins by colon carcinoma cells has been correlated with poor prognosis (73), and increased metastatic potential (74, 75, 76). Previous work has shown that these highly glycosylated molecules participate in the initial weak adhesion or "tethering" of circulating cells to activated endothelium (41, 71, 77). Specifically, the numerous carbohydrate side chains (ie. sLe^x & sLe^a) of mucins are recognized and bound by endothelial selectins (78 - 81).

Two additional mucin molecules, CD43 (leukosialin) (42) and mucosal addressin cell adhesion molecule (MAdCAM-1) (80), have also been suggested to negotiate adhesion to molecules involved in the firm attachment phase of circulating cell—endothelial cell adhesion. MAdCAM-1, found on high endothelial venules, binds L selectin (80), and $\alpha_4\beta_7$ integrins (82). CD43, expressed on T cells, a subset of B cells, and other hematopoietic cells, has been shown to bind ICAM-1(42, 83). The binding of CD43 to ICAM-1 is interesting in that CD43 shows no structural resemblance to β_2 integrins (CD11a/b combined with CD18, also known as LFA-1, and Mac-1); the major ligands for ICAM-1.

Normal mucin structure



Carcinoma mucin structure

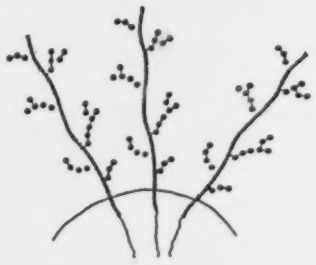


Figure 5. Cancer associated mucins differ from their normal counterparts in that they show increased expression over the entire cell surface and are underglycosylated thereby exposing normally cryptic carbohydrate and peptide epitopes.

1.5. The Mucin, MUC1 and its association with Cancer

Mucins are expressed on the surface of many benign and malignant epithelial cells. A major member of the mucin family is MUC1, also known as polymorphic epithelial mucin, epithelial membrane antigen, human milk fat globule membrane antigen etc. exists in a wide variety of secretory epithelia of human organs. These include pancreas, lung, kidney, oviduct, salivary gland, uterus, stomach, and mammary gland. It has also been shown to be overexpressed and aberrantly glycosylated in several epithelial cancers and in over 90 % of breast cancers (84).

Antigenic differences between tumor associated mucins and their normal cell counterparts exist due to altered patterns of glycosylation (77, 85) (Figure 6), and subtle changes in the protein backbone (86). Exploitation of these differences is an active area of research which includes the use of tumor associated MUC1 epitopes as candidate targets for active specific immunotherapy (87), and in specific targetting of cytotoxic drugs to tumor cells. In addition, secreted tumor MUC1 detected by mAbs serves as a serum tumor marker for disease progression.

1.5.1. Structure of MUC1

The human MUC1 gene maps to region 21q on chromosome 1, a region frequently found to be altered in cancer cells (88). It encodes the core protein of MUC1 which exhibits the features of an integral membrane protein, consisting of three distinct domains (Figure 7).

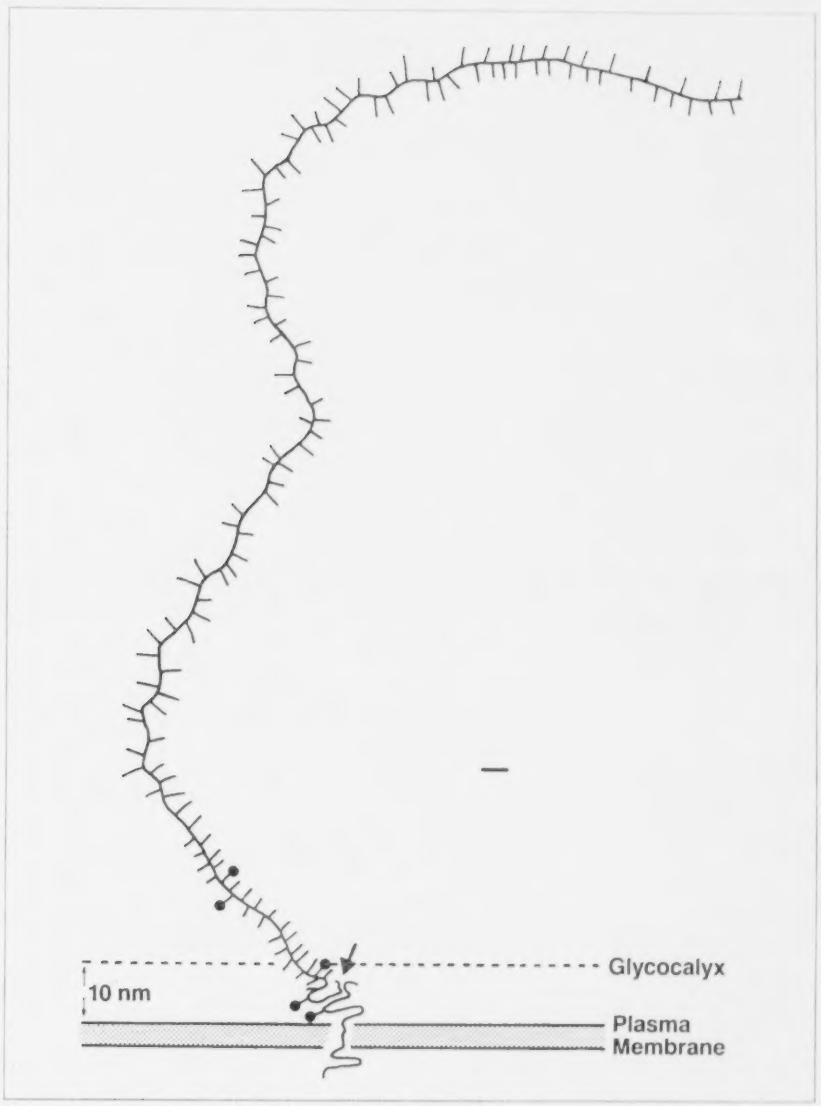


Figure 6. The extended and rigid structure of mucins allow them them to protrude far above the surrounding glycocalyx. (taken from ref. 72)

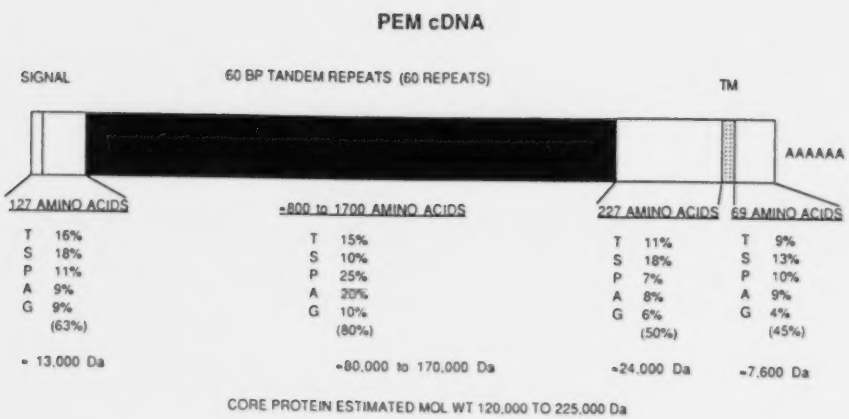


Figure 7. The mucin MUC1shows the structure of an integral membrane protein with short cytoplasmic and transmembrane domains followed by an extended extracellular domain including a signal peptide at its amino terminus. (taken from ref. 88)

The majority of the protein (middle region) is made up of a variable number of tandem repeat (VNTR) sequences of 20 amino acids. The number of tandem repeats varies from 30 - 90 in the population with the number for each individual determined by an inherited genetic polymorphism. Each repeat contains two serines, and three threonines. Four of these five amino acids occur as doublets, corresponding to what might be expected for an extensively glycosylated protein. Separating these potential glycosylation sites are "spacer" regions rich in prolines. The protein's amino terminus (outermost region) is composed of a hydrophobic signal peptide and degenerate tandem repeats. Its carboxy terminus (transmembrane and cytoplasmic regions) consists of degenerate tandem repeats leading into another hydrophobic sequence consistent with a 31 amino acid transmembrane domain. This is followed by a 69 amino acid cytoplasmic tail which is linked to microfilaments of the cytoskeleton (89).

As many as one in four amino acids may be glycosylated in MUC1 (88). The majority of these sugars are O-linked and many contain sialic acid residues, giving them a strong negative charge. Differences between normal and tumor associated MUC1 can be largely attributed to differential glycosylation. Tumor cell MUC1 often differs both in abundance and patterns of glycosylation. This variability may occur due to incomplete or neosynthesis depending on the expression or organization of glycosyltransferases in the cell. Also, substrate availability and competition contribute to carbohydrate diversity. Some novel carbohydrates seen in tumor mucins are sialyl Le^a, sialyl Tn, TF, etc. which are expressed on epithelia only during fetal development and in tumors (90).

1.5.2. Function(s) of MUC1

Knowledge of the physiological function(s) of MUC1 is limited. The differences in structure and extremely high levels of MUC1 expression exhibited by cancer cells suggest there may be additional functions to the protective and / or lubricative roles of MUC1 in normal epithelia. Normal glandular epithelia tend to show a restricted membrane pattern where MUC1 expression is limited to the luminal "free membrane" (Figure 8a). In breast carcinomas there is often a ten fold or greater increase in the amount of MUC1 and this is variably distributed throughout the cytoplasm, intracellular lumina, and over the entire cell surface (72, 91) (Figure 8b). This dramatic difference in expression, coupled with aberrant glycosylation suggest that MUC1 may be important in the neoplastic process.

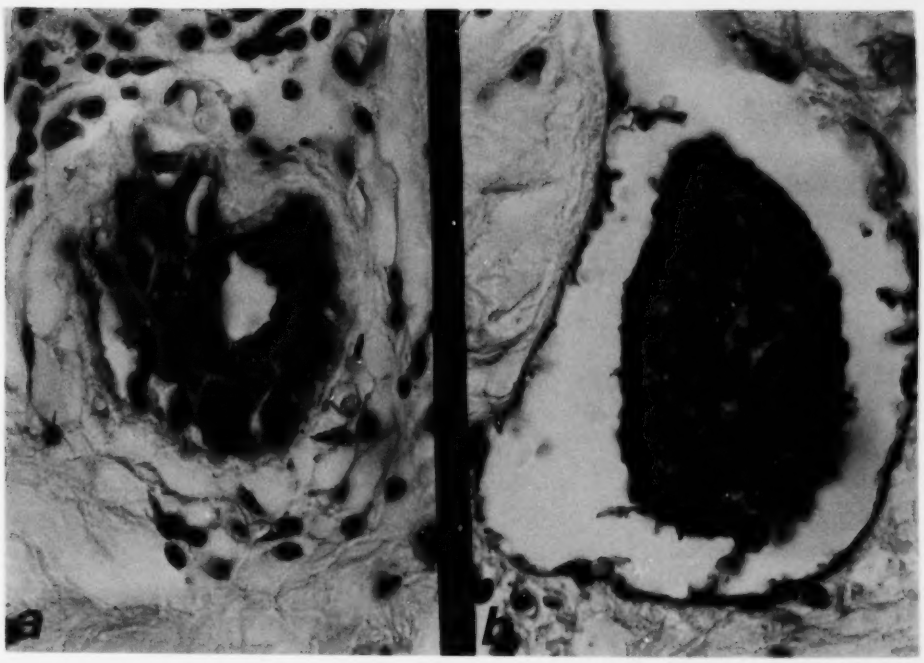


Figure 8. Benign mammary epithelia **(a)** stained for MUC1 using the DAB immunoperoxidase technique shows an apical (luminal) staining pattern in contrast to an intravascular tumor embolus **(b)** which shows intense peripheral staining. Note the uneven staining along the endothelial surface **(b)** which likely represents shed antigen. (x400 original magnification)

a). MUC1 as an Anti - Adhesive Molecule

In vitro data showing reduced cellular aggregation (92), and reduced cytotoxic T cell killing (93) of cells expressing a high density of MUC1 has prompted some investigators to suggest that MUC1 serves as an anti-adhesion molecule. The propsed anti-adhesion mechanism is based on a masking effect of MUC1 over the much shorter adhesion molecules within the glycocalyx. Due to its rigid structure, MUC1 extends to a length of 200 - 300 nm, far beyond the surrounding glycocalyx at 10 - 30 nm (72). Additionally, its high number of sialylated carbohydrate residues form an outer perimeter cloud of negative charge creating a repulsive effect (72).

b). MUC1 as a Pro - Adhesive Molecule

The extended and rigid structure of MUC1 places it in an ideal position to facilitate adhesion to cells possessing the appropriate counter-receptors. MUC1 and similar mucins have been demonstrated to facilitate adhesion to selectins by virtue of their numerous accessible sialylated carbohydrates (ie. sLe^x, sLe^a) (71, 94 - 96).

1.6. Hypothesis

The data presented in this thesis extends the adhesive capability of MUC1, suggesting that it binds ICAM-1. As ICAM-1 is such a widespread and important adhesion molecule, it is plausible that tumor cells might express a receptor for ICAM-1. The fact that tumor cells rarely express the commonly accepted ICAM-1 ligands (β_2 integrins), and the finding that a similar mucin, CD43 (leukosialin) has been shown to bind ICAM-1 are in agreement with the suggested MUC1—ICAM-1 interaction. MUC1 is rarely expressed on normal

MLC. As such, it offers a potential target for anti-metastatic therapy without compromising inflammatory cell migration.

1.7. Purpose of this Study

This study aims to identify the major adhesive interactions between breast carcinoma cells and endothelial cells.

Initially, a human breast carcinoma cell line, MCF-7 and human endothelial cells of two types, HUVEC and immortalized hybrids of HUVEC, and lung carcinoma (A549) cells, EAhy926, were used in a standard *in vitro* static adhesion assay.

In the first half of chapter 2 of this thesis, the following will be addressed:

1. Expression of endothelial adhesion molecules, E selectin, P selectin, ICAM-1, and VCAM-1 will be determined following 0, 4, & 24 h of cytokine stimulation.

2. Adhesive function blocking mAbs to endothelial adhesion molecules E selectin, P selectin, ICAM-1, and VCAM-1 will be used to determine endothelial adhesion mechanisms facilitating MCF-7 adhesion.
3. mAbs to MCF-7 surface molecules sLe^x, sLe^a, sTn, and MUC1 peptide core will be used to determine contribution of these molecules to MCF-7 - endothelial cell adhesion.

The second half of chapter 2 will explore the possibility of the breast cancer mucin MUC1 mediated adhesion to the immunoglobulin superfamily member, ICAM-1, and will address the following:

1. Adhesion to human endothelium of murine breast adenocarcinoma cells (410.4) which are negative for MUC1 will be compared with two 410.4 cell lines transfected with the human MUC1 gene. The corresponding adhesion molecules will be confirmed by mAb adhesion inhibition.

2. Adhesion of MUC1 expressing cancer cells to murine lymphoma cells (BW57) transfected with either human E selectin, ICAM-1, or CD36 and inhibition of adhesion using mAbs will be observed.

3. Adhesion of MUC1 expressing cancer cells to immobilized human ICAM-1 fusion proteins and blockage of adhesion using mAbs and soluble MUC1 will be studied to determine the sufficiency of ICAM-1 in MUC1 mediated adhesion.

4. The requirement for metabolic energy and divalent cations in the MUC1—ICAM-1 interaction will be assessed in adhesion of murine MUC1 transfected cells to EAhy926 stimulated for 4 h.

Chapter 2

EXPERIMENTAL METHODS AND RESULTS

2.1. Phenotypical analysis of Endothelia

Objective:

To phenotype the HUVEC and EAhy926 cell line for the expression of endothelial adhesion molecules; E selectin, P selectin, ICAM-1, & VCAM-1 following 0, 4, & 24 h cytokine stimulation.

Materials and Methods:

mAbs

Monoclonal anti-E selectin (1.2B6), and anti-ICAM-1 (84H10) were obtained from Serotec Canada. Monoclonal anti-P selectin (AK-6), and anti-VCAM-1 (1.G11B1) were obtained from Southern Biotechnology Associates Inc.

Cells

Cultured endothelial hybrid cell line EAhy926 was kindly provided by Dr. Cora Jean S. Edgell, Department of Pathology, University of North Carolina at Chapel Hill, and was maintained in RPMI with 10% FCS. Human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection and were maintained in Ham's F12K with 10% FCS, 30 ug/mL endothelial cell growth supplement (Sigma), and 100 ug/mL heparin.

Reagents

Recombinant human cytokines Interleukin-1 (IL-1β), and Tumor Necrosis Factor (TNFα) were obtained from Cedarlane Laboratories Canada (Pharmingen).

Endothelial Cell Stimulation

Endothelial cells between passages 2 and 5 were cultured in 8 well tissue culture slides (Nunc Inc.) to confluence before addition of cytokines. 20 U/mL of IL-1β

and 20 U/mL of TNFα in endothelial cell growth media was added to the PBS washed endothelial monolayers and the slides were incubated for 4 or 24 h at 37° C.

Immunocytochemical Phenotyping

After cytokine stimulating endothelial cell slide cultures, the monolayers were again washed with PBS. The slides were left to airdry overnight and the following day were tested for expression of various adhesion molecules using the two step indirect immunoperoxidase technique. Primary mouse monoclonal antibodies (at previously determined optimal concentrations - see Table 1) were incubated with cytospin slides for 40 min at room temperature in a humidified chamber. After washing with PBS, a secondary peroxidase conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.) at 30 ng/mL was added for 30 min under similar conditions. Peroxidase staining was obtained using the following peroxidase substrate solution: 9 mL PBS + 0.2 mL 30% hydrogen peroxide + 0.8 mL 70 mg/mL 3,3'-diaminobenzidine (DAB) for 10 min. Cells were counterstained with Harris hematoxylin for 1 min and rinsed in warm water for 1 min. Slides were then coverslipped using Permount. Adhesion molecule expression was graded from negative (neg) to 4+ where 1+ corresponds to 1-19% positive cells, 2+ = 20-49%, 3+ = 50-74%, and 4+ = 75-100%.

Flow Cytometric Phenotyping

Cultured cells were harvested using 0.05% (w/v) trypsin with 0.53 mM EDTA and were washed and suspended at 1 x 10^6 / mL in PBS. Cell suspensions were stained using the two step indirect immunofluorescence technique. Primary mouse monoclonal antibodies (at previously determined optimal concentrations -

Table 1) were incubated with cell suspensions for 40 min on ice. After washing with PBS, secondary phycoerythrin (PE) conjugated goat anti-mouse antibodies (Southern Biotechnology Associates Inc.) at 2 ng/mL, were incubated with cells for 30 min on ice and in the dark. Stained cells (10,000 / analysis) were then analyzed within 1 h of staining on a FACScan (Becton Dickinson).

Table 1.

Antibody Concentrations or Dilutions used in Experimental Methods

Antibody	Method	Concentration or Dilution
1.2B6 (anti-E selectin)	Imm. cytochem.	50 ug/mL
"	Flow cytom.	33 ug/mL
"	Adhesion blockade	20 ug/mL
AK-6 (anti-P selectin)	Imm. cytochem.	25 ug/mL
"	Flow cytom.	20 ug/mL
"	Adhesion blockade	20 ug/mL
84H10 (anti-ICAM-1)	Imm. cytochem.	50 ug/mL
"	Flow cytom.	33 ug/mL
"	Adhesion blockade	20 ug/mL
1.G11B1 (anti-VCAM-1)	Imm. cytochem.	25 ug/mL
"	Flow cytom.	20 ug/mL
"	Adhesion blockade	20 ug/mL
18E3D (anti-ICAM-1)	Adhesion blockade	20 ug/mL
164B (anti-ICAM-1)	Adhesion blockade	20 ug/mL
B67.4 (anti-sLea)	Adhesion blockade	20 ug/mL
CSLEX (anti-sLex)	Adhesion blockade	20 ug/mL
B72.3 (anti-sTn)	Adhesion blockade	20 ug/mL
B27.29 (anti-MUC1)	Imm. cytochem.	0.94 ug/mL
"	Flow cytom.	0.94 ug/mL
"	Adhesion blockade	20 ug/mL
DF3P (anti-MUC1)	Adhesion blockade	20 ug/mL
All control antibodies (CD31, VIIIra, EMA, Moc31)	Adhesion blockade	20 ug/mL

Results:

Basal (unstimulated)

a). The EAhy926 cell line showed only a modest level (1+) of ICAM-1 expression. E & P selectins, and VCAM-1 were not detected (Table 2).

b). The HUVEC also showed ICAM-1 as the only adhesion molecule inherently present (1+), while the selectins, and VCAM-1 were absent (Table 3).

4 hours stimulation

a). At 4 h of stimulation the EAhy926 cells displayed only minimal expression of E selectin (1+), while P selectin remained undetected. VCAM-1 was slightly upregulated (1+) and ICAM-1 was greatly increased (from 1+ to 4+) (Table 2).

b). In contrast, the HUVEC showed a substantial increase in E selectin (4+) and ICAM-1 (4+) expression following an identical 4 hour cytokine treatment. P selectin (1+), and VCAM-1 (1+) expression was only minimally increased following stimulation (Table 3).

24 hours stimulation

a). Following 24 h of stimulation E & P selectins, had returned to their basal undetectable levels on the EAhy926. However, ICAM-1 persisted at high levels (3+) and VCAM-1 was slightly higher than at 4 h (2+) (Table 2).

b). Selectins were not detected on the HUVEC at 24 h post stimulation. ICAM-1 was highly expressed (4+), and VCAM-1 was also slightly higher than at 4 h (2+) (Table 3).

Table 2.

EAhy926 Adhesion Molecule Expression by Immunocytochemistry

Molecule	Unstim.	4 hr cytokine stim.	24 hr cytokine stim.
E selectin	neg.	1+	neg.
P selectin	neg.	neg.	neg.
ICAM-1	1+	4+	3+
VCAM-1	neg.	1+	2+

Table 3.

HUVEC Adhesion Molecule Expression by Immunocytochemistry

Molecule	Unstim.	4 hr cytokine stim.	24 hr cytokine stim.
E selectin	neg.	4+	neg.
P selectin	neg.	1+	neg.
ICAM-1	1+	4+	4+
VCAM-1	neg.	1+	2+

2.2. Implicated Molecules in MCF-7 Breast Carcinoma cells to Endothelia

Objective

To determine the relative involvement of endothelial (selectins, ICAM-1, & VCAM-1) and breast carcinoma (sLe^x, sLe^a, sTn, & MUC1) cell associated adhesion molecules in the *in vitro* adhesion of the human breast carcinoma cell line, MCF-7 to cytokine (IL-1β & TNFα) stimulated endothelial monolayers. Both EAhy926 endothelial hybrids and HUVEC were used at 4 and 24 h post stimulation.

Materials and Methods:

mAbs

Monoclonal anti-MUC1 (B27.29, & DF3P), anti-sTn (B72.3), anti-sLe^a (B67.4), and anti-sLe^x (CSLEX) were kindly provided by Biomira Inc.

Cells

Cultured human breast carcinoma cell line MCF-7 was a gift from Biomira Inc. and was maintained in DMEM with 10% (v/v) FCS, 2mM glutamine, and 0.1 mg/mL insulin.

Tumor Cell - Endothelial Cell Adhesion Assay

Endothelial monolayers between passage 2 and 5 were grown to confluence in 24 well culture plates (Costar) and were stimulated with 20 U/mL TNFα + 20 U/mL IL-1β (lowest concentrations required for optimal adhesion enhancement as determined in preliminary work) for 4 or 24 h. Monolayers were then gently washed with PBS to remove cytokines. To these plates BCECF-AM ester (Molecular Probes) fluorescent dye labelled tumor cells (1.5×10^5/500uL/well) were added and incubated for 25 min at 37° C. Non - specifically adherent tumor cells were removed by physical agitation (shaker plate at 175 rpm) and

peripheral aspiration. Remaining adherent cells were then lysed with detergent (NP-40) for 30 min and the percent adhesion (dye signal) was quantitated using a SPEX fluorimeter and compared to and expressed as a percentage of the signal from the total number of cells added to each well (Figure 9). For antibody inhibition experiments, to determine the relevant endothelial adhesion molecules, endothelial cells were pretreated with monoclonal antibodies at 30 ug/mL in growth medium or with medium alone, for 30 min at 37° C. To determine the relevant tumor cell adhesion molecules, tumor cells were pretreated with monoclonal antibodies at 20 ug/mL in DMEM with 2% (v/v) FCS or with medium alone (no antibody) for 30 min at room temperature before carrying out adhesion assays. Concentration of mAbs, temperature, and treatment duration described above were similar to those in the literature (4, 5). Results are expressed as the mean percent adhesion of 3 seperate wells +/- SD. Statistical analysis was performed using the t test and statistical significance was defined as $p < 0.05$.

Tumor cell/ endothelial cell adhesion assay

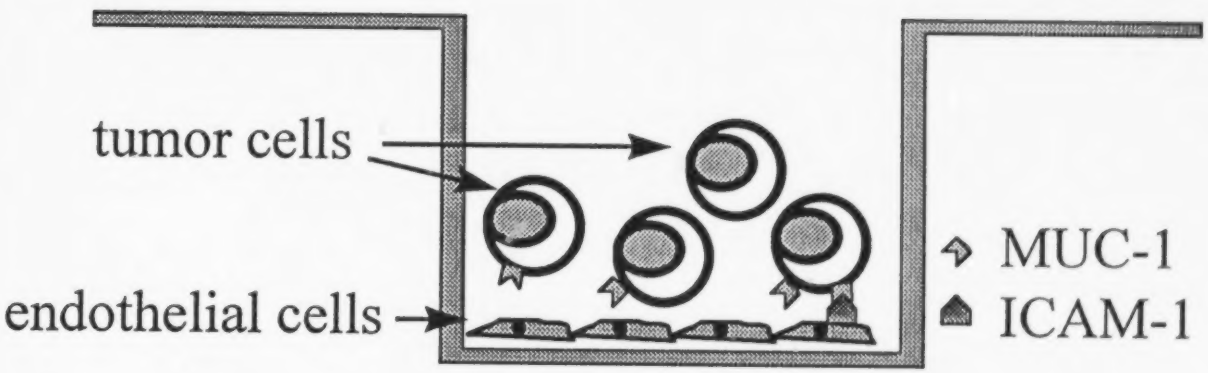


Figure 9. The tumor cell - endothelial cell in vitro adhesion assay consisting of tissue culture wells coated with confluent endothelial monolayers onto which tumor cell suspensions are added and co - incubated for 25 minutes before gently washing and quantitating the adherent tumor cells.

Results:

4 hours stimulation (mimics acutely stimulated endothelium)

Adhesion of MCF-7 to EAhy926 was increased by approximately 1.8 fold following 4 h cytokine stimulation. Pretreatment of the EAhy926 monolayers with mAbs against endothelial adhesion molecules (Figure 10) showed that antibodies against the selectins and against ICAM-1 abrogated the adhesion enhancement following the 4 h stimulation. MCF-7 pretreatment with mAbs to sLe^x, sLe^a, and sTn demonstrated adhesion inhibition shown in Figure 11. Antibodies to the sialylated Lewis antigens ($sLe^{x/a}$) blocked 66% of the cytokine enhanced adhesion. In addition, mAbs to the peptide core of MUC1 (B27.29) (inhibited 96% of adhesion enhancemenct) and to one of its carbohydrate side chains, sialylated Tn (B72.3), (inhibited 90% of adhesion enhancement) were also potent inhibitors of MCF-7 adhesion.

Adhesion of MCF-7 to HUVEC was increased by approximately 2.4 fold following 4 h of cytokine stimulation. Antibodies to E selectin (endothelial monolayer pretreatment) or its ligands ($sLe^{x/a}$) (MCF-7 pretreatment) showed the greatest inhibition of adhesion (Figures 12 & 13). An antibody to ICAM-1 (84H10) also caused inhibition but much less than that obtained by anti-E selectin (1.2B6). An antibody to MUC-1 (B27.29) had no effect.

24 hours stimulation (mimics chronically stimulated endothelium)

Adhesion of MCF-7 to EAhy926 stimulated for 24 hr was only enhanced by 1.2 fold over that to unstimulated monolayers. Pretreatment of endothelial monolayers with mAbs showed that anti-ICAM-1 completely abrogated the cytokine enhanced adhesion while antibodies to the selectins and to VCAM-1 showed approximately 60% inhibition (Figure 14). Pretreatment of the MCF-7

cells with antibodies to MUC1 inhbited the enhanced adhesion by approximately 190% (Figure 14), while no other antibodies to carcinoma antigens inhibited adhesion.

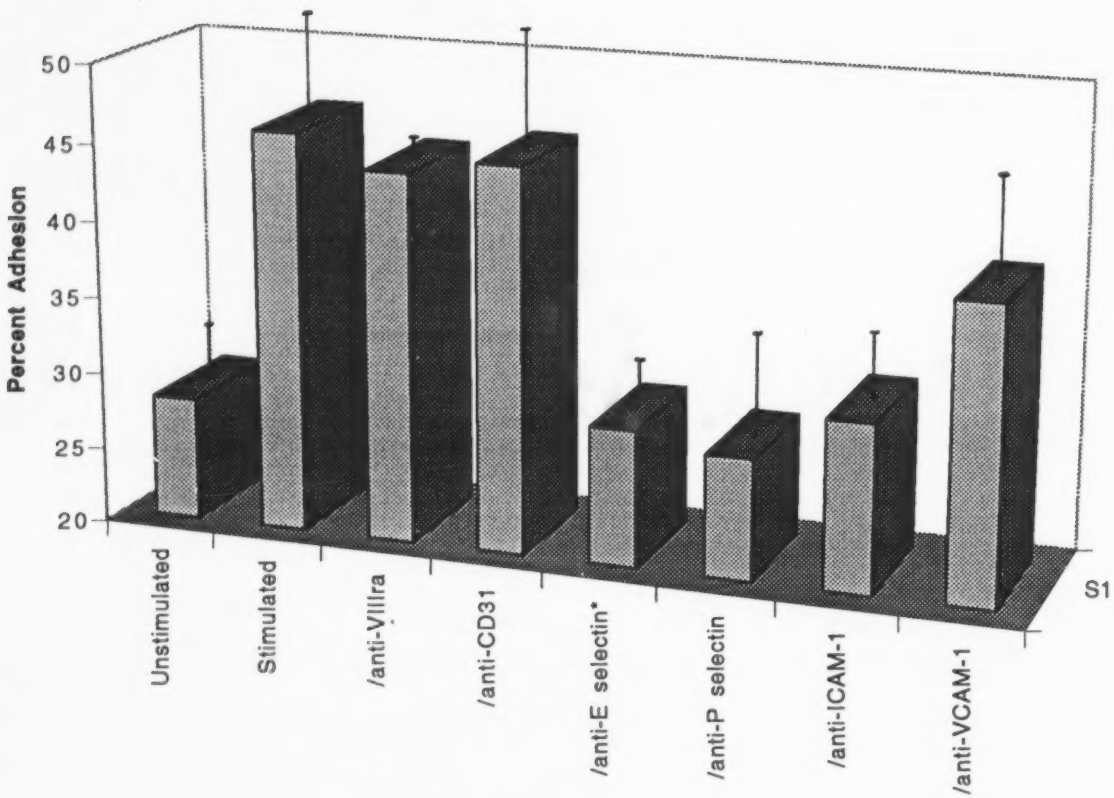


Figure 10. Adhesion of MCF-7 cells to 4 h stimulated EAhy926 cells in the presence of mAbs to endothelial adhesion molecules. /, refers to wells coated with stimulated monolayers. *, represents statistical significance.

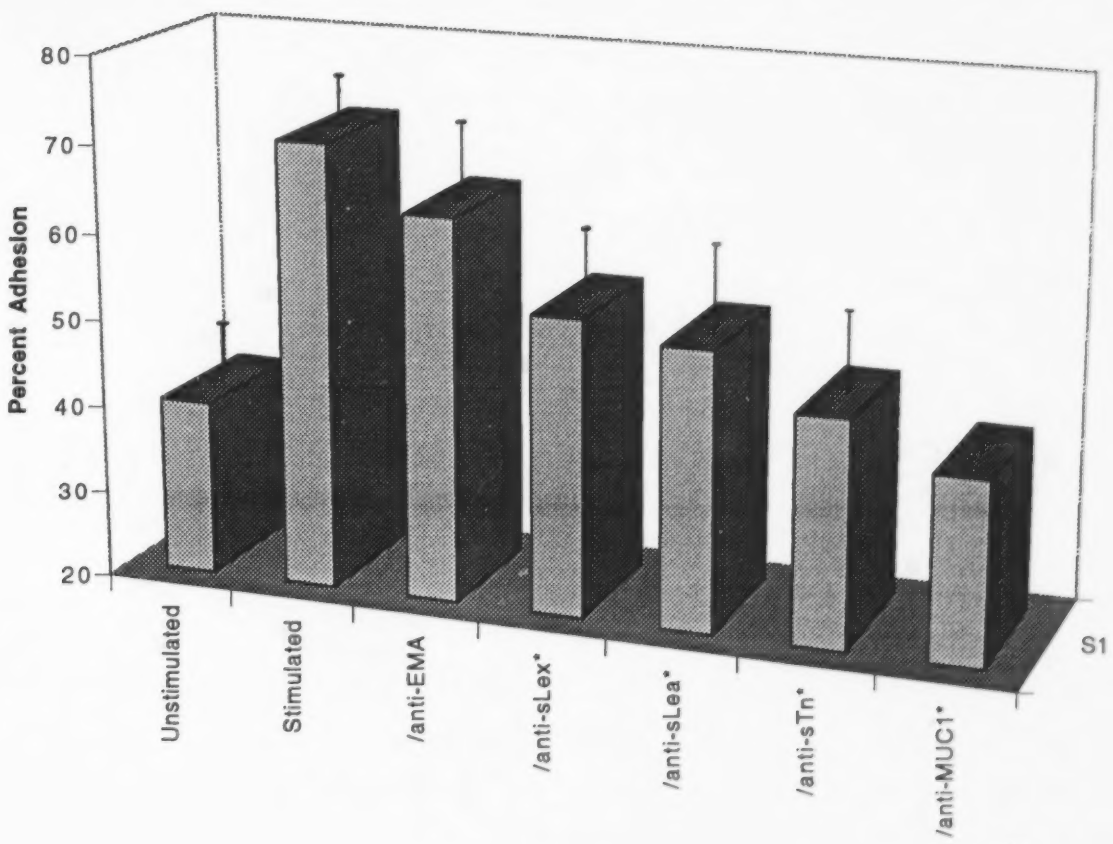


Figure 11. Adhesion of MCF-7 cells to 4 h stimulated EAhy926 cells in the presence of mAbs to MCF-7 adhesion molecules. /, refers to wells coated with stimulated monolayers. *, represents statistical significance.

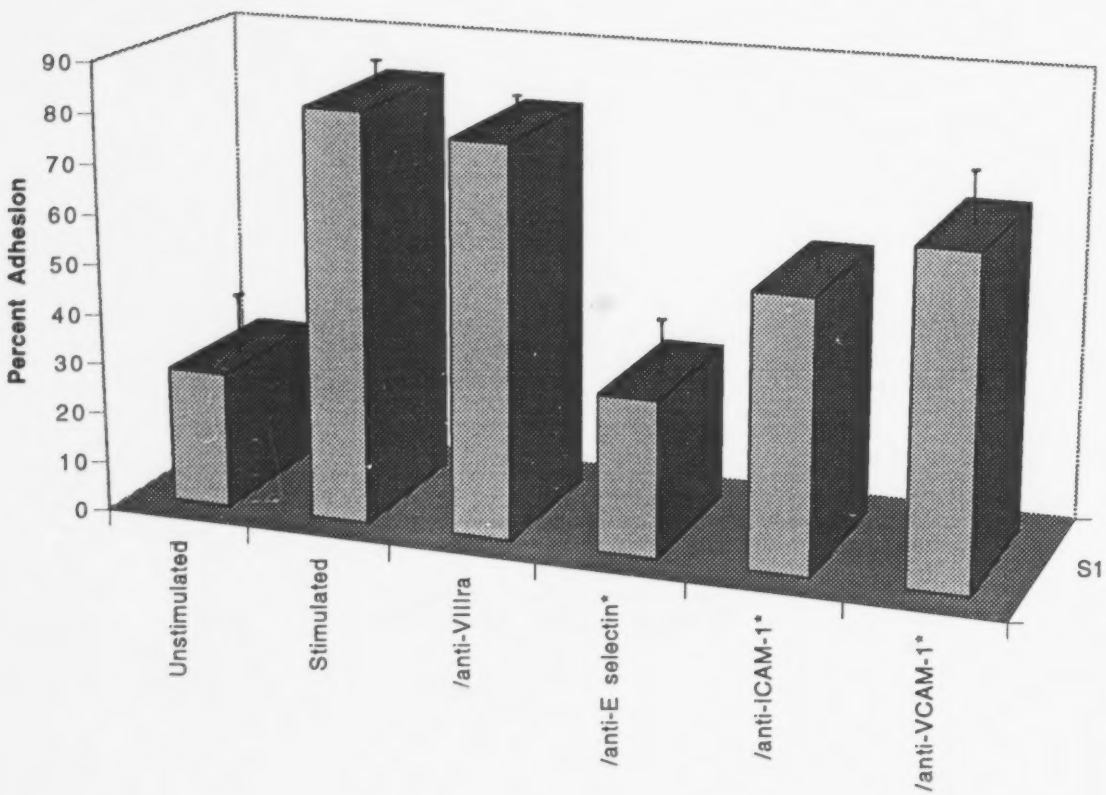


Figure 12. Adhesion of MCF-7 cells to EAhy926 cells to 4 h stimulated HUVEC in the presence of mAbs to endothelial adhesion molecules. /, refers to wells coated with stimulated monolayers. *, represents statistical significance.

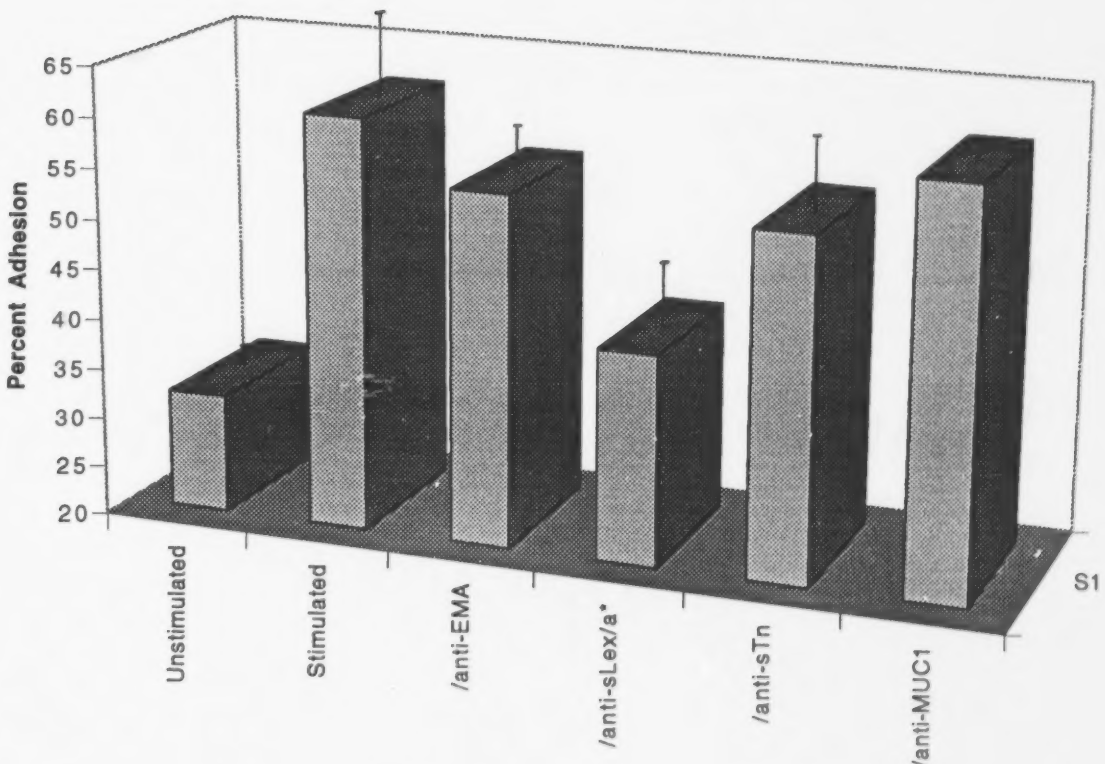


Figure 13. Adhesion of MCF-7 cells to 4 h stimulated HUVEC in the presence of mAbs to MCF-7 adhesion molecules. /, refers to wells coated with stimulated monolayers. *, represents statistical significance.

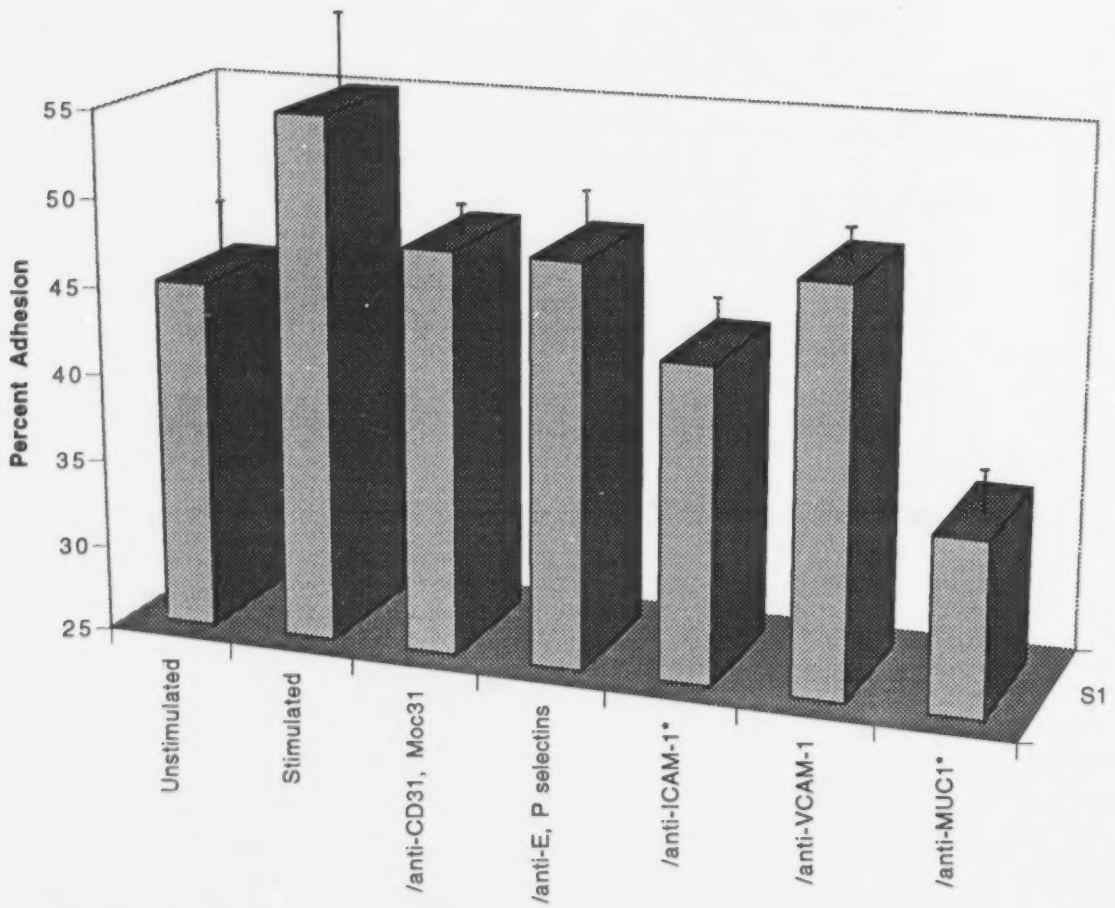


Figure 14. Adhesion of MCF-7 cells to 24 h stimulated EAhy926 cells in the presence of mAbs to endothelial and MCF-7 adhesion molecules. /, refers to wells coated with stimulated monolayers. *, represents statistical significance.

Enhancement of MCF-7 adhesion to HUVEC at 24 h was increased by about 1.7 fold over unstimulated conditions. In contrast to results obtained with HUVEC stimulated for 4 h, antibodies to E selectin and its ligands $sLe^{x/a}$ showed little to no inhibition of MCF-7 adhesion to 24 h stimulated HUVEC (Figure 15). There was substantial inhibition (120%) of enhanced adhesion by anti-ICAM-1 (84H10) pretreatment of HUVEC. Using the same system, MCF-7 cells were pretreated with various mAbs. Only the mAb to MUC1 (B27.29) consistently blocked the adhesion of MCF-7 to HUVEC at 24 h (Figure 15).

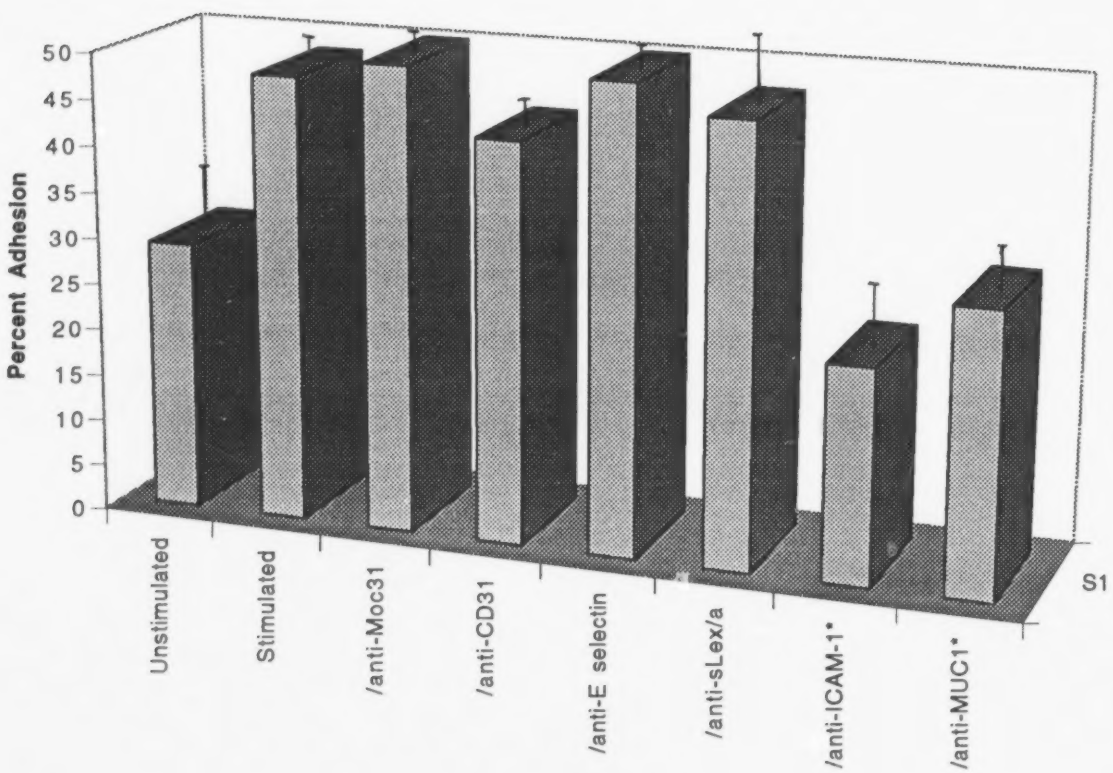


Figure 15. Adhesion of MCF-7 cells to 24 h stimulated HUVEC in the presence of mAbs to endothelial and MCF-7 adhesion molecules. /, refers to wells coated with stimulated monolayers. *, represents statistical significance.

2.3. Summary of 2.1. and 2.2.

The hybrid endothelial - lung carcinoma cell line, EAhy926 differs from HUVEC in that it shows only a minimal elevation of E selectin expression after 4 hours of stimulation while HUVEC shows heavy expression. Both cell types show a greatly increased level of ICAM-1 expression at 4 and 24 hours post stimulation.

i). MCF-7 adhesion to 4 h stimulated HUVEC is mediated by selectin based mechanisms. The MUC1 core protein is not involved.

ii). MCF-7 adhesion to 4 and 24 h stimulated EAhy926 and to 24 h stimulated HUVEC is inhibited by antibodies to MUC1 and to ICAM-1.

These data suggest that the interaction of MUC1 and ICAM-1 may be a novel tumor cell—endothelial cell adhesion mechanism active subsequnet to selectin based adhesion.

2.4. Involvement of MUC1 in Endothelial Adhesion

Objective:

To substantiate the role for MUC1 in tumor cell—endothelial cell adhesion.

Materials and Methods:

mAbs

Monoclonal anti-ICAM-1 antibodies (18E3D, 164B, 164I) were kindly provided by Dr. Michael Gallatin at ICOS corp.

Cells

Cultured murine breast carcinoma cell line 410.4 (wild type), and human *MUC1* gene transfectants of these: GZLo (low MUC1 expressor) & GZHi (high MUC1

expressor) were the generous gifts of Dr. Michael Longenecker and were maintained in DMEM with 10% (v/v) FCS. As quantitated by flow cytometry, the GZHi transfectants showed approximately the same level of MUC1 expression as that of the human breast carcinoma line, MCF-7 (data not shown). This MUC1 density was 2.5 times higher than that of GZLo while the wild type parental cell line was negative (Figure 16). These results were confirmed by immunohistochemistry. Flow cytometric analysis revealed that the GZHi cell line was negative for the established ligands of ICAM-1, β_2 integrins and CD43 (Figure 17).

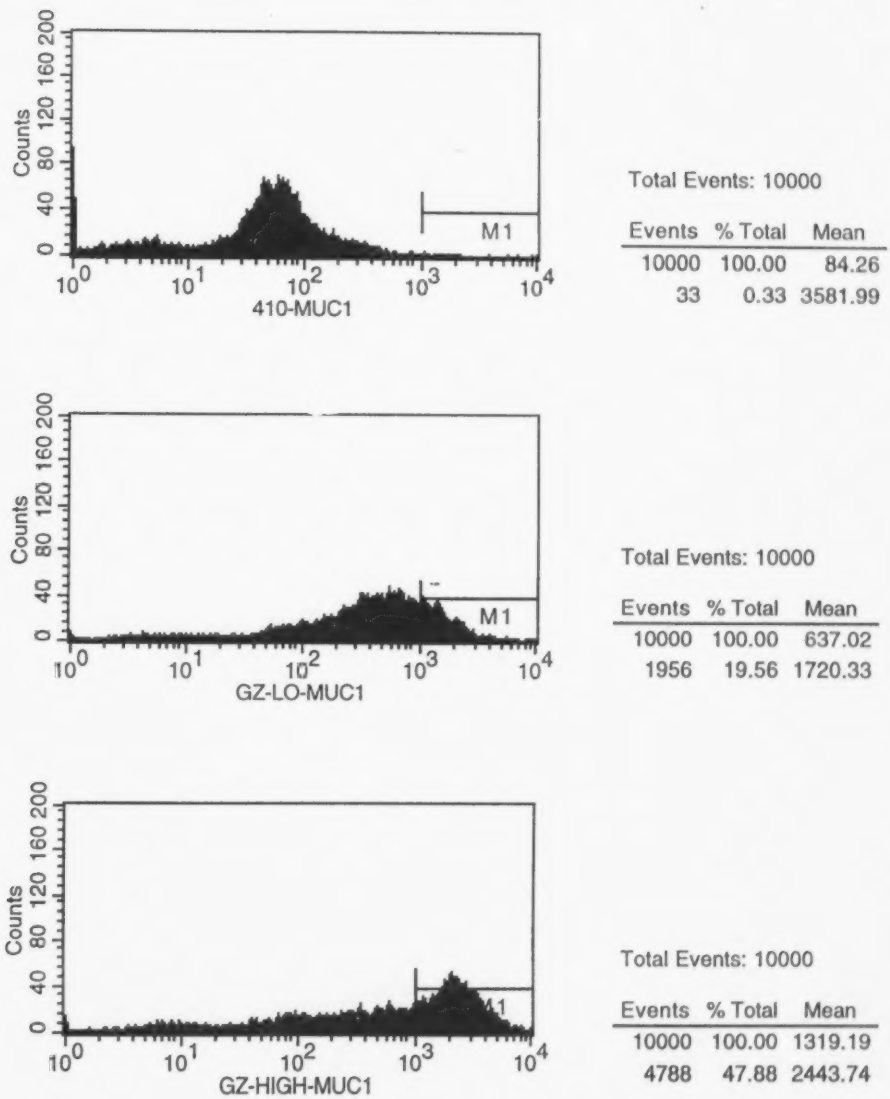
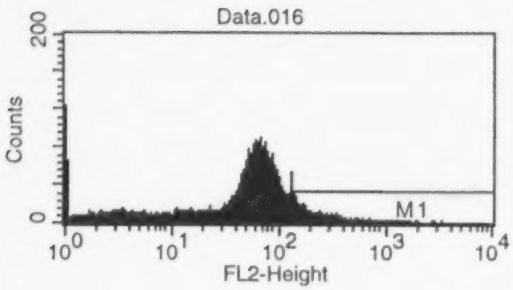


Figure 16. MUC1 expression by murine 410.4 cells (wild type) and low (GZLo), and high (GZHi) MUC1 expressing transfectants as ascertained by flow cytometry.

GZHi - CD43

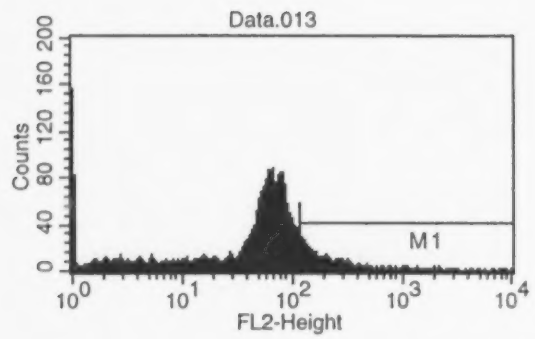


Histogram Statistics

File: Data.016 Gated Events: 10000
Total Events: 10000

Events	% Gated	% Total	Mean
10000	100.00	100.00	74.99
944	9.44	9.44	284.83

GZHi - LFA-1

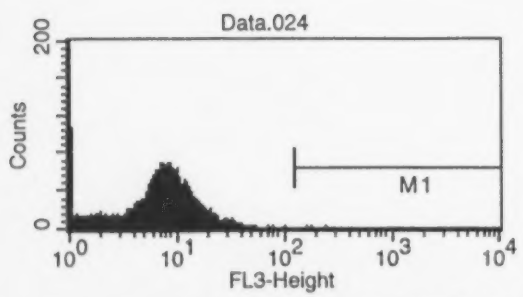


Histogram Statistics

File: Data.013 Gated Events: 10000
Total Events: 10000

Events	% Gated	% Total	Mean
10000	100.00	100.00	100.68
1412	14.12	14.12	394.57

GZHi - Mac-1



Histogram Statistics

File: Data.024 Gated Events: 10000
Total Events: 10000

Events	% Gated	% Total	Mean
10000	100.00	100.00	7.58
3	0.03	0.03	182.65

Figure 17. Lack of CD43 and β_2 integrin expression by GZHi as ascertained by flow cytometry.

MUC1 Transfected Cell - Endothelial Cell Adhesion Assay

Murine 410.4 mammary adenocarcinoma cells transfected with the human MUC1 gene showing either low (GZLo) or high (GZHi) surface expression of tumor like MUC1 were compared to the MUC1 negative wild type (410.4) for adhesion to 4 h stimulated EAhy926 and 24 h stimulated HUVEC using the *Tumor Cell—Endothelial Cell Adhesion Assay* described in section 2.2. The effect of pretreatment with mAb to endothelial (ICAM-1 and E selectin) and tumor cell (MUC1) antigens was also perfomed and examined as in section 2.2.

Results:

GZHi to 24 h stim. HUVEC: GZHi exhibited four times greater adhesion to 24 h stimulated HUVEC as compared to wild type (410.4), and 2.6 fold greater adhesion than GZLo (see Figure 18). Antibody to MUC1 (B27.29), as well as an antibody to ICAM-1 (18E3D) blocked adhesion of GZHi to 24 h stimulated HUVEC (Figure 19) almost completely, while anti-E selectin and a control antibody to CD31 showed no inhibitory effect.

GZHi to 4 h stim. EAhy926: Adhesion of GZHi to EAhy926 at 4 h (Figure 20) was substantially inhibited (67% of enhancement) by mAb to MUC1 (B27.29).

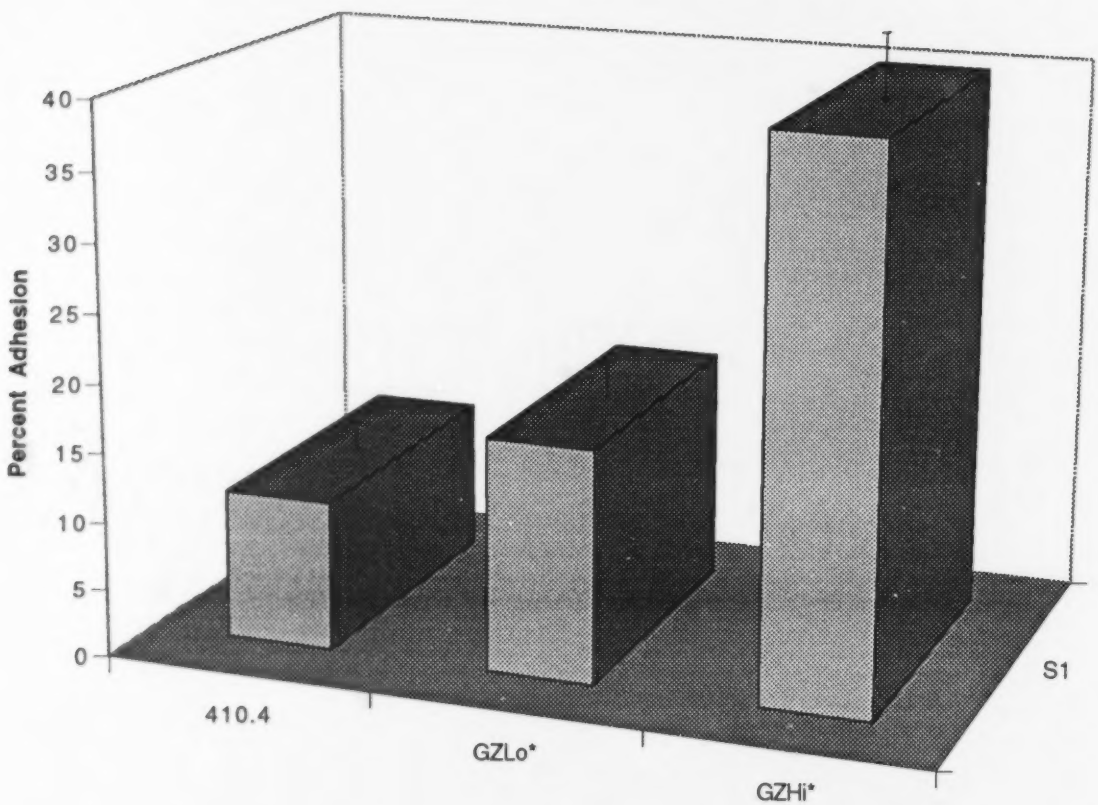


Figure 18. Adhesion of Murine 410.4 cells and human MUC1 transfectants of these to 24 h stimulated HUVEC and correlation to level of MUC1 expression. *, represents statistical significance.

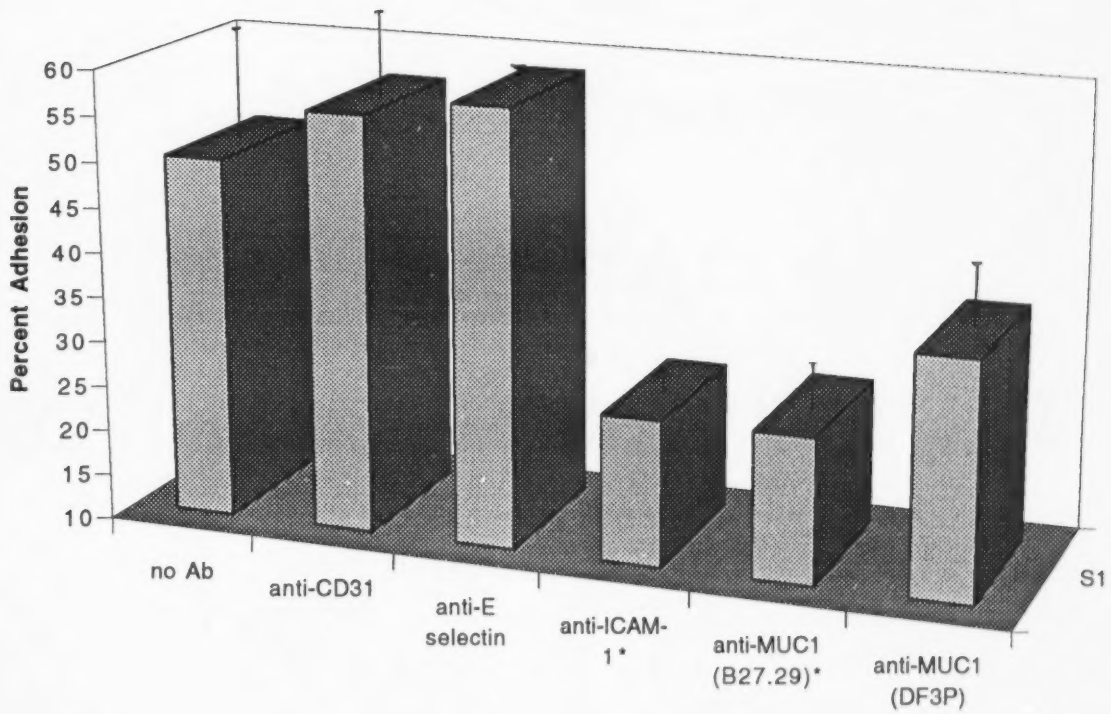


Figure 19. Adhesion of GZHi cells to 24 h stimulated HUVEC in the presence of mAbs to endothelial adhesion molecules and to MUC1. *, represents statistical significance.

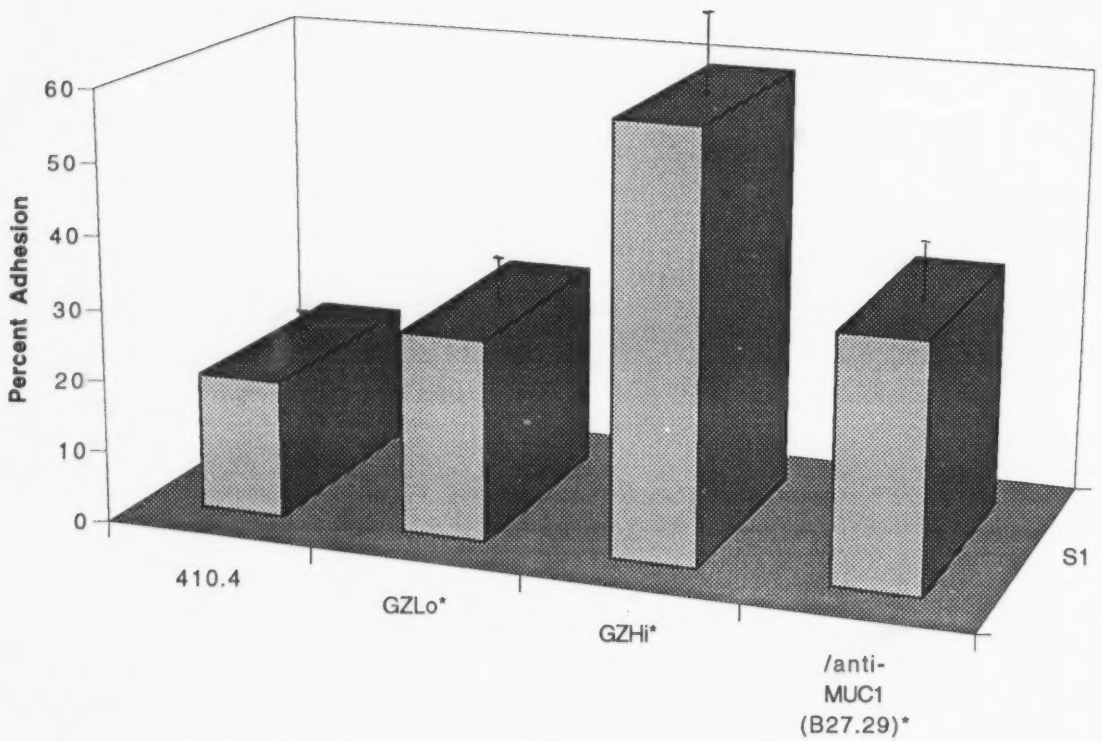


Figure 20. Adhesion of Murine 410.4 cells and human MUC1 transfectants of these to 4 h stimulated EAhy926 cells and correlation with level of MUC1 expression. /, refers to GZHi pretreated with mAbs. *, represents statistical significance.

2.5. Involvement of ICAM-1 in MUC1 mediated Adhesion

Objective:

To demonstrate that the endothelial ligand for MUC1 is ICAM-1.

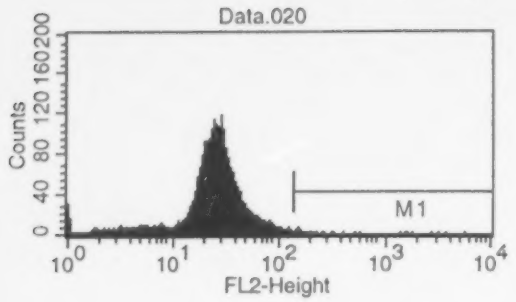
Materials and Methods:

Cells

Cultured murine lymphoma cell line BW/57 (wild type), and human E selectin (BW/ELAM), ICAM-1 (BW/ICAM), and CD36 (BW/CD36) transfectants of these were kindly donated by Dr. John Elliott and were maintained in RPMI 1640 with 10% (v/v) FCS. The BW/57 cells were analyzed for expression of L selectin, a known adhesion ligand for mucins and their sialylated carbohydrate side chains. This antigen was not detected on these cells (Figure 21).

Suspended Cell Aggregation Assay

2.5×10^5 MUC1 transfected cells (GZHi) were added to a similar number of BW57 cells transfected with and expressing human CD36 (BW/CD36), E selectin (BW/ELAM), or ICAM-1 (BW/ICAM). Cells were suspended in RPMI 1640 with 2% (v/v) FCS and allowed to aggregate on a shaker (120 rpm) for three hours at room temperature. 250 uL aliquots were aspirated at 0, 1, 2, & 3 h and analyzed using an automated cell counter (Coulter Electronics). The extent of aggregation was determined using the formula No/Nt, where No is the number of single cells and or small aggregates at 0 hours and Nt is the number at subsequent sample times. With increasing aggregation the value of Nt decreases (and thus the No/Nt index increases) giving a positive correlation between No/Nt and adhesion. In the corresponding antibody inhibition experiments, antibody pretreatments of the cells were carried out at room temperature for 30 min prior to mixing of the two adhesion partners.

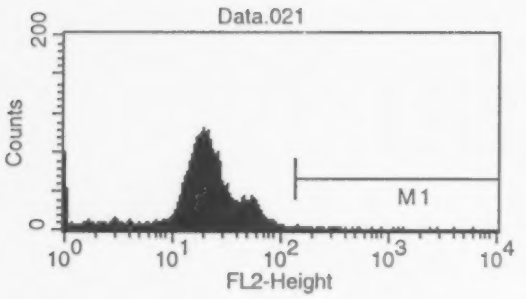


BW/CD36 - L selectin

Histogram Statistics

File: Data.020 Gated Events: 10000
Total Events: 10000

Events	% Gated	% Total	Mean
10000	100.00	100.00	31.05
70	0.70	0.70	629.04

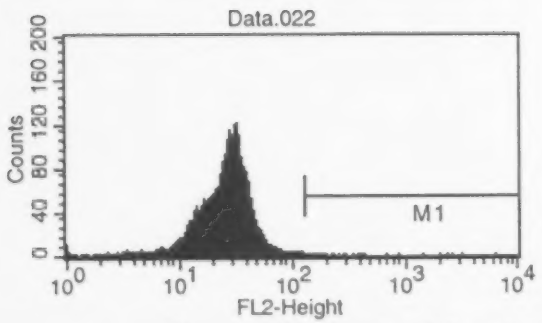


BW/ELAM - L selectin

Histogram Statistics

File: Data.021 Gated Events: 10000
Total Events: 10000

Events	% Gated	% Total	Mean
10000	100.00	100.00	27.33
65	0.65	0.65	551.00



BW/ICAM - L selectin

Histogram Statistics

File: Data.022 Gated Events: 10000
Total Events: 10000

Events	% Gated	% Total	Mean
10000	100.00	100.00	30.07
44	0.44	0.44	590.42

Figure 21. Lack of L selectin expression on three BW/57 murine cell lines transfected with human CD36, E selectin, or ICAM-1 as ascertained by flow cytometry

Results:

Aggregation between GZHi and BW/ICAM was approximately three times greater than that of the heterotypic control, GZHi and BW/CD36, and homotypic controls (Figure 22). The aggregation between GZHi and BW/ICAM could be abolished by preincubating the corresponding cells with mAbs to MUC1 (B27.29) and to ICAM-1 (84H10) (Figure 22). Aggregation of GZHi with BW/ELAM showed an intermediate value in subsequent experiments (Figure 23) likely due to the sialylated carbohydrate content of MUC1.

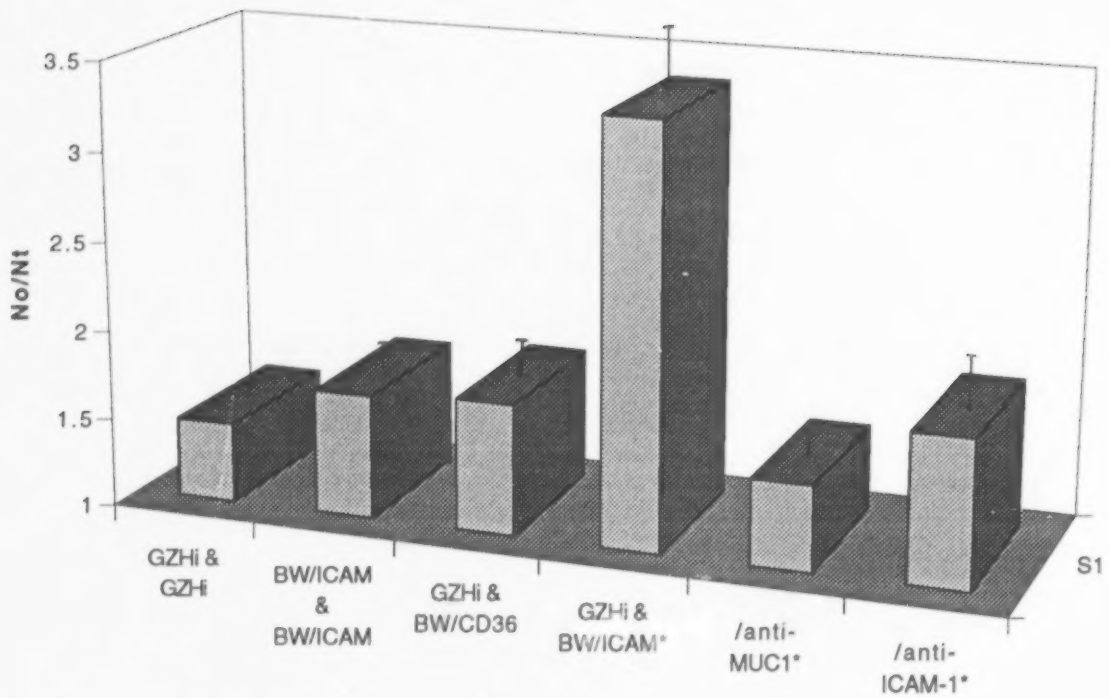


Figure 22. Aggregation of GZHi cells with human CD36, or ICAM-1 transfected BW/57 cells. No is the number of single cells and/or small aggregates at time zero, while Nt is the number following 3 h of aggregation. /, refers to pretreatment of GZHi or BW/ICAM with mAbs. *, represents statistical significance.

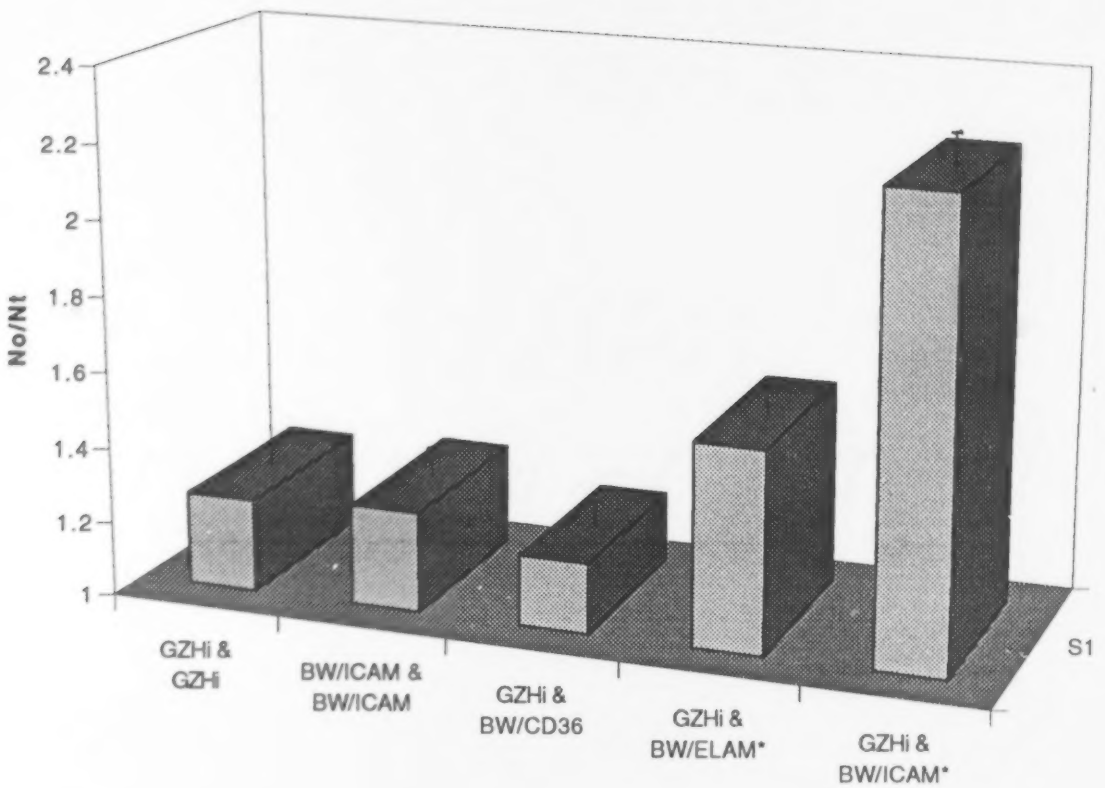


Figure 23. Aggregation of GZHi cells with human CD36, E selectin, and ICAM-1 transfected BW/57 cells. *, represents statistical significance.

2.6. Sufficiency of ICAM-1 in MUC1 mediated Adhesion

Objective:

To exclude the possibility that adhesion was being mediated by other endothelial adhesion molecules in addition to ICAM-1.

Materials and Methods:

Reagents

Soluble human MUC1 was obtained from ascites of breast cancer patients, purified using an immunoaffinity column labelled with monoclonal anti-MUC1 (B27.29) and was a kind gift of Biomira Inc.

Tumor Cell—Immobilized ICAM-1 Adhesion Assay

The adhesion of MUC1 expressing cells to immobilized recombinant human ICAM-1—Ig fusion protein was studied. Adhesion to rhICAM-1was compared to Bovine Serum Albumin (BSA) and Collagen type I as controls using the following protocol. 96 well tissue culture plates were coated with 50 uL of a solution with 20 ug/mL of soluble recombinant human ICAM-1 - Ig fusion protein in PBS for one hour at room temperature. Collagen type I control wells were coated with 50 uL of a solution with 10 ug/mL of collagen type I (Celltrix Vitrogen 100) in 10 nM HCL. Wells were then blocked with 2% (w/v) BSA for two h at 37° C and washed four times with PBS. A 30 uL volume of antibodies (10ug/mL) (anti-ICAM-1 & anti-E selectin) or purified soluble MUC1 (10 ug/mL) were added to the wells for 90 min at room temperature. Wells were then washed again before addition of tumor cells. Fluorimetric dye labelled tumor cells were pretreated at room temperature with 20 ug/mL of anti-MUC1 in DMEM with 2% (v/v) FCS or with medium alone (no antibody) and were then added to the appropriate (1 x 10^5/well) wells for 40 min at 37° C.

Antibody and soluble MUC1 pretreatments used are similar to those in the literature (4, 5, 97). Wells were then washed and percent adhesion was determined as described in the *Tumor cell—Endothelial cell adhesion assay* in section 2.2.

Results:

Wild type (410.4), GZLo, and GZHi cells displayed similarly low binding to 2% (w/v) BSA coated wells. GZHi showed approximately 3.5 fold higher binding to rhICAM-1 coated wells than did 410.4 and GZLo (Figure 24). Anti-MUC1 (B27.29) and anti-ICAM-1 (18E3D) antibodies successfully abrogated the increased adhesion of GZHi to rhICAM-1. These MUC1 antibodies did not however, inhibit high levels of adhesion by MUC1 expressing cells to a collagen type I control in subsequent experiments (Figure 25).

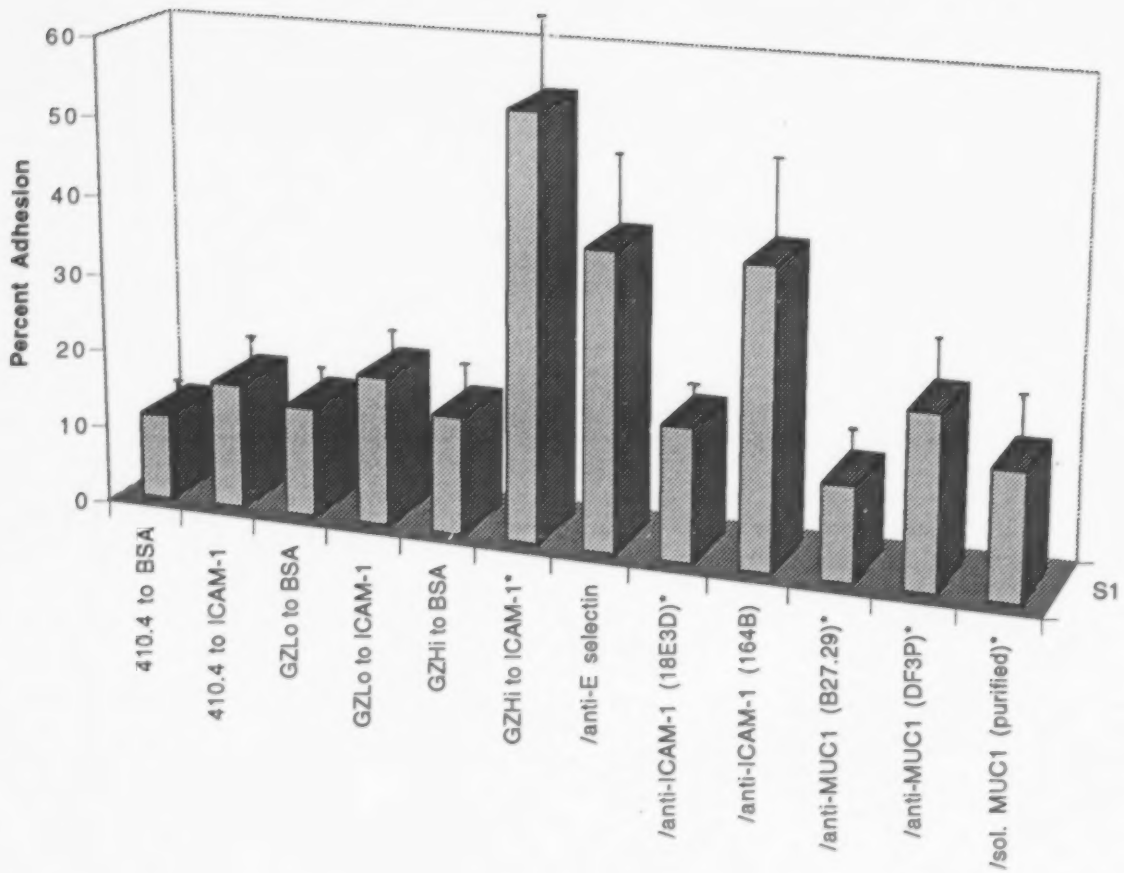


Figure 24. Adhesion of Murine 410.4 cells and human MUC1 transfectants of these to immobilized ICAM-1 in the presence of purified MUC1 and mAbs to ICAM-1 and to MUC1. /, refers to GZHi adhesion to ICAM-1. *, represents statistical significance.

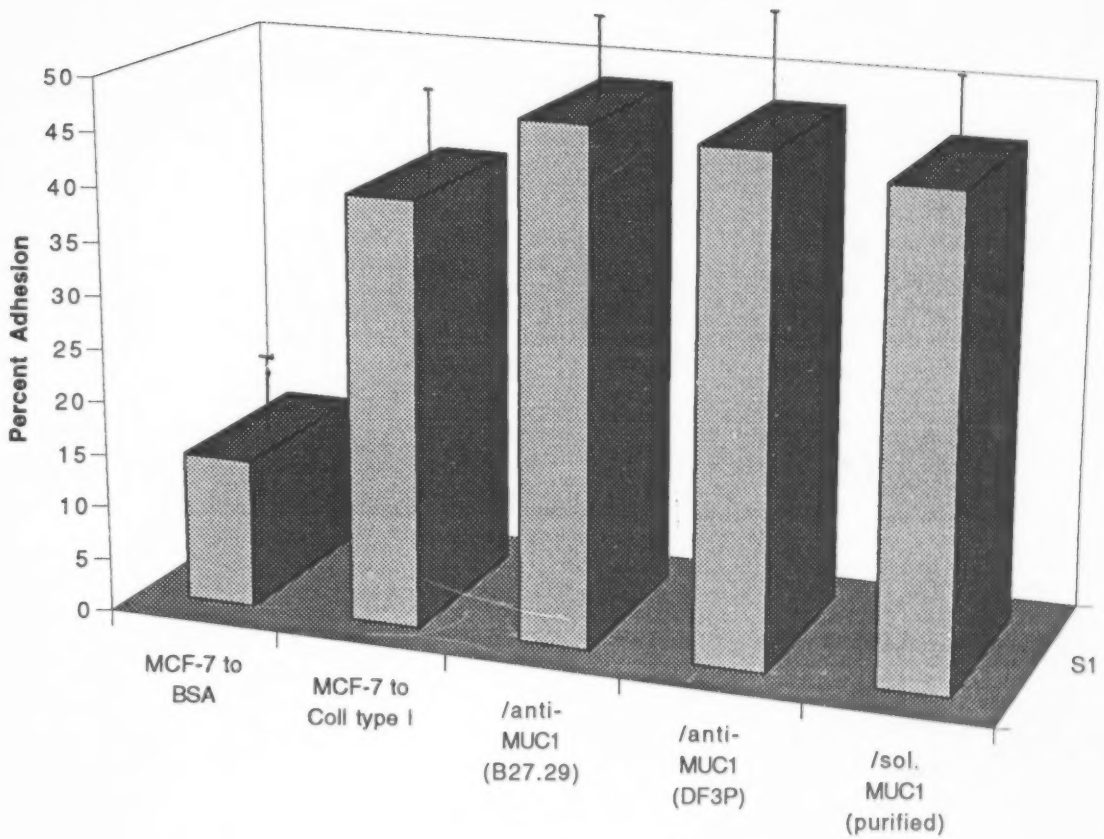


Figure 25. Adhesion of MCF-7 cells to Collagen type I in the presence of purified MUC1 and mAbs to MUC1. /, refers to MCF-7 adhesion to collagen type I.

2.7. Importance of Tumor Secreted MUC1

Objective:

To investigate whether free MUC1 can bind to ICAM-1 and provide competition for cell surface bound MUC1.

Results:

Purified MUC1 blocked adhesion of the MUC1 transfectants to immobilized rhICAM-1 as effectively as anti-ICAM-1 (18E3D) (Figure 24), however it did not affect adhesion to collagen type I controls (Figure 25).

2.8. Characterization of the MUC1—ICAM-1 Interaction

Objective:

This study examines the MUC1—ICAM-1 interaction for divalent cation requirements and temperature dependence.

Materials and Methods:

Characterization of MUC1—ICAM-1 interaction for energy and divalent cation requirements

Determination of energy and divalent cation requirements was carried out using the tumor cell (GZHi)—endothelial cell (EAhy926) adhesion assay. The EAhy926 were prepared as in Part I and used after 4 h of cytokine stimulation. Fluorimetric dye labelled GZHi cells were added and co - incubated at 37° C for 25 min before aspiration of non - adherent cells, and lysing and determination of adherent cell fraction.

a). Temperature dependence experiments were carried out at 4° C, room teperature, and 37° C in the absence of mAbs or any other adhesion modulating agents.

b). Divalent cation requirement experiments were conducted using media in which cations were titrated into the system as Ca^{2+} alone, Mg^{2+} alone, and Ca^{2+} plus Mg^{2+}. Divalent cations were added as $CaCl_2$ and / or $MgCl_2$ salts at concentrations of 0, 0.1, 1.0, & 10 mM. mAbs or other adhesion modulating agents were not present.

Results:

2.8.1. Temperature Dependence

The adhesion between GZHi and EAhy926 was temperature dependent with optimal binding seen at 37° C. Adhesion was reduced to less than 10% of the value obtained at 37° C when the assay was conducted at 4° C (Figure 26). An intermediate level of adhesion was observed at room temperature.

2.8.2. Divalent Cation Dependence

Adhesion between GZHi and EAhy926 was optimized by addition of 1mM Mg^{2+}. Addition of Ca^{2+} alone at similar concentrations supported less than 1/3 of the maximal adhesion. The presence of both cations at 1mM each supported an intermediate level of approximately 1/2 of the maximal adhesion seen with Mg^{2+} only (Figure 27).

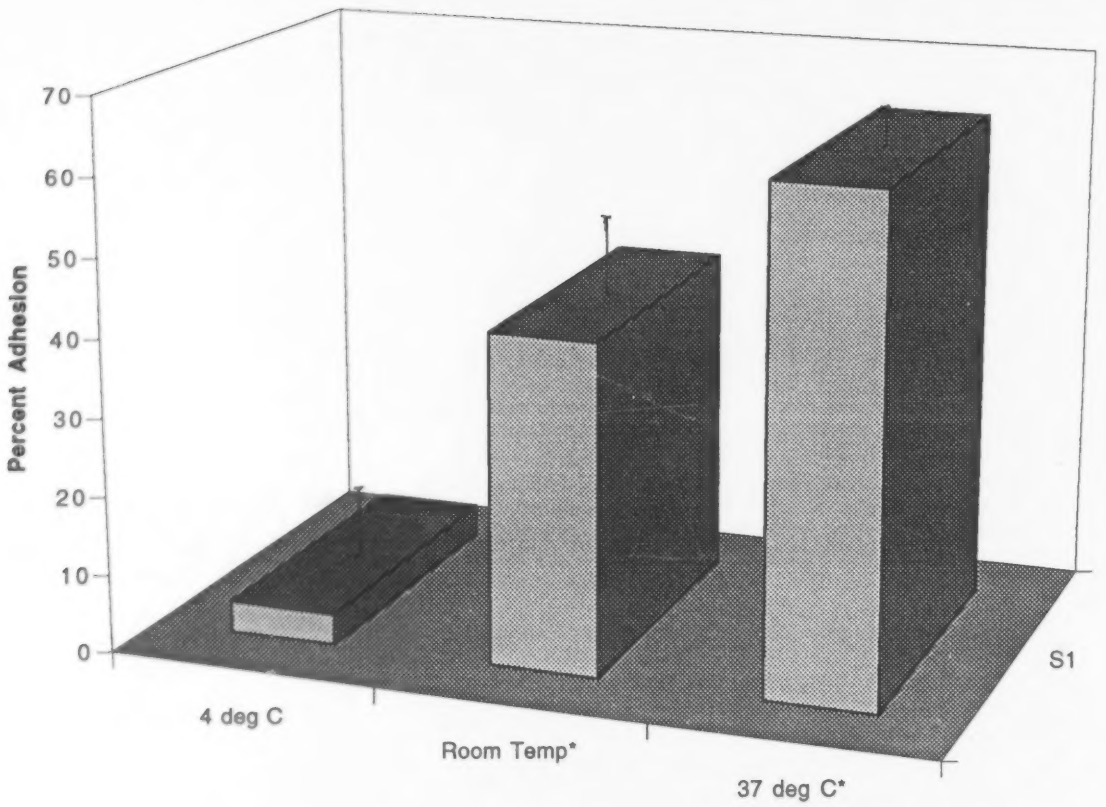


Figure 26. Temperature Dependence of GZHi cell adhesion to 4 h stimulated EAhy926. As previously demonstrated, this adhesion is largely MUC1—ICAM-1 mediated. *, represents statistical significance.

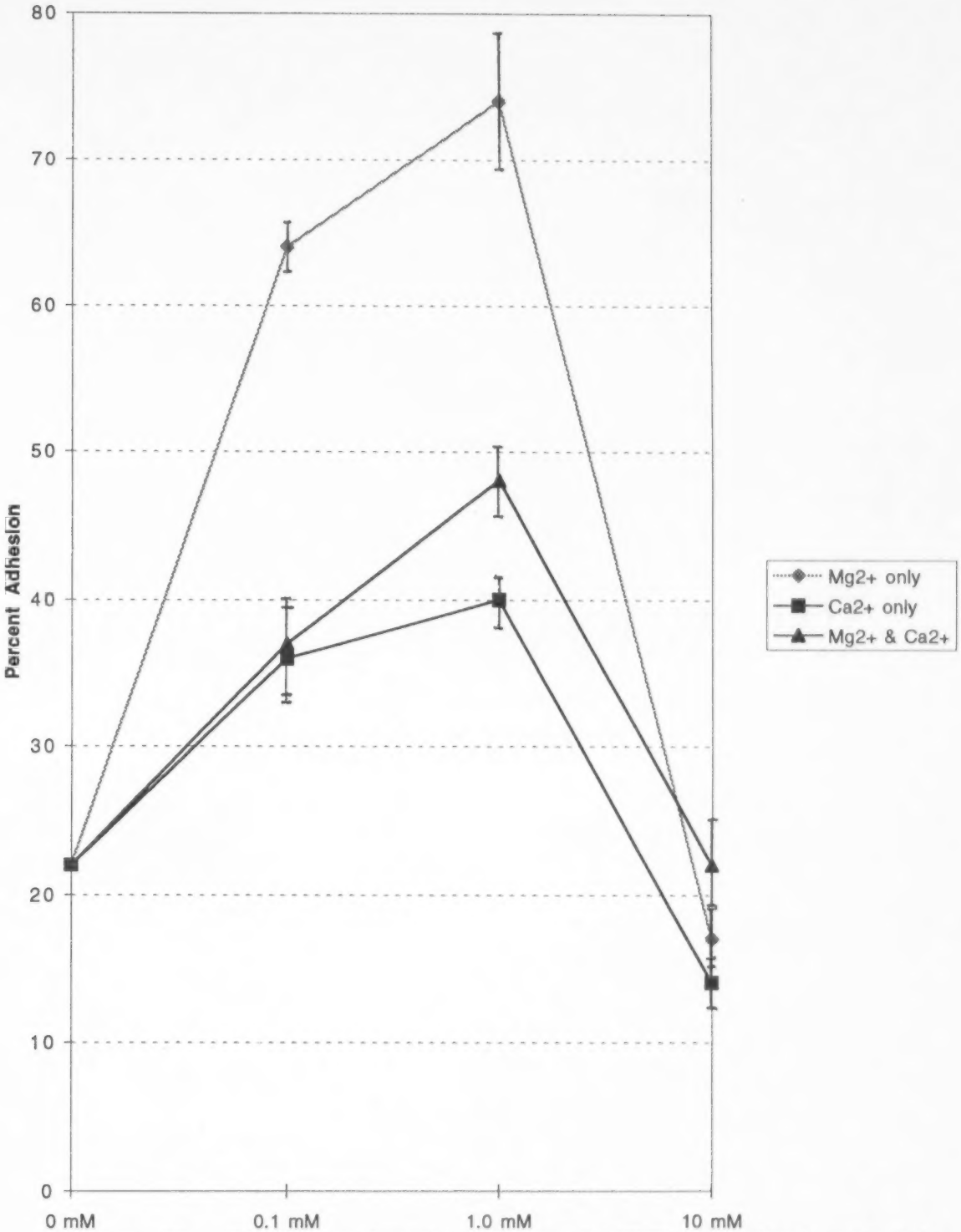


Figure 27. Divalent cation dependence of GZHi cell adhesion to 4 h stimulated EAhy926.

2.9. Summary of 2.3.-2.8.

i). Increased adhesion to ICAM-1 expressing endothelium correlates with increasing MUC1 expression by tumor cells.

ii). Aggregation of GZHi with BW/57 cells is greatest with ICAM-1 transfected BW/57 cells.

iii). High MUC1 expressing cells bind specifically to rhICAM-1 coated plates. Soluble MUC1 competes with its cell bound counterpart for ICAM-1 binding.

iv). MUC1 / ICAM-1 interaction is an active energy requiring process.

v). The MUC1 / ICAM-1 interaction requires the presence of divalent cations with Mg^{2+} being the optimal cation and Ca^{2+} being able to competitively inhibit enhanced adhesion by Mg^{2+}.

In summary, the data suggest that the MUC1—ICAM-1 interaction is an active, and specific interaction requiring the presence of Mg2+ and metabolic energy.

Chapter 3

DISCUSSION AND CONCLUSIONS

3.1. Introduction

A very important feature of a malignant cell is its ability to metastasize through the vascular system. A crucial step in the process of metastasis consists of adhesive interactions between the circulating tumor cells and the endothelial cell lining of the blood vessels at sites of secondary tumor spread. There is evidence that tumor cells may utilize the receptor—ligand interactions that normal inflammatory cells use to migrate through the vascular endothelial cell layer. Therapeutic manipulation of these interactions would carry unacceptable morbidity as normal immune function might be seriously compromised. This thesis presents functional evidence for a novel mechanism of tumor cell—endothelial cell adhesion using a unique antigen which is relatively specific for epithelial cancers and is expressed on over ninety percent of breast cancers.

Circulating cell—endothelial cell adhesion is thought to occur via two classes of adhesion molecule interactions. Selectin—carbohydrate interactions are responsible for the initial weak adhesion or "rolling", while immunoglobulin superfamily—integrin interactions allow for subsequent firm attachment and transendothelial migration.

3.2. Review and discussion of Experimental Data

As demonstrated in section 2.1., the adhesion molecule profile of cytokine stimulated HUVEC varies over time. E selectin is expressed early with a maximum peak at 4 h post stimulation and is gradually lost by 24 h. ICAM-1 is also expressed at 4 h, however unlike selectins, it persists at a high density for at least 24 h. The endothelial cell hybrid, EAhy926 shows a consistent elevation of ICAM-1 with little E selectin expression at both 4 and 24 h post stimulation. As described in section 2.2., Tumor cell MUC1 showed involvement in adhesion to

HUVEC only after 24 h of stimulation and to EAhy926 after both 4 and 24 h of stimulation. While E selectin is present only at low levels in these three situations, endothelial ICAM-1 is highly expressed. This provided the first functional evidence that MUC1 may be an additional tumor mucin type ligand for ICAM-1.

In the subsequent experiments there was a linear relationship between the levels of MUC1 expression and adhesion to 24 h stimulated HUVEC as mentioned in section 2.4., and shown in figures 16 & 18. This direct proportionality supported the hypothesis that MUC1 itself was the relevant ligand binding to stimulated endothelium. This is confirmed by the substantial adhesion inhibition seen following tumor cell pretreatment with anti-MUC1 (B27.29).

To rule out the possibility that high MUC1 expressing cells are non - specifically "sticky" to several cell types regardless of their adhesion molecule profiles, the high MUC1 expressing transfectant, GZHi was tested for adhesion to murine lymphoma cells transfected with human ICAM-1, E selectin or CD36 (section 2.5.) The BW/57 cell lines do not adhere to cell culture wells and so a dynamic assay involving adhesion in suspension was used. The use of this second assay system excludes any bias which may be inherent to the previously used static adhesion system. As figures 22 & 23 show, the greatest adhesion occurs with the ICAM-1 transfectant. Specificity of the interaction was again confirmed by adhesion inhibition by anti-MUC1 (B27.29), and anti-ICAM-1 (18E3D). This experiment confirmed ICAM-1 as the adhesion molecule recognized by MUC1.

The cancer associated MUC1 apomucin has been shown to contain sLe^x (95, 96) and sLe^a (96) carbohydrate side chains which are known ligands for E selectin.

This likely accounts for the intermediate level of adhesion seen between GZHi and the E selectin transfectant, BW/ELAM in figure 23.

The sufficiency of ICAM-1 as the only endothelial adhesion molecule required to facilitate MUC1 based adhesion was demonstrated by studies of adhesion of MUC1 transfected cells to immobilized human recombinant ICAM-1 - Ig fusion protein (section 2.6.) (see Figure 24). These showed specific adhesion of high MUC1 expressing cells to ICAM-1 which was inhibited by antibodies to MUC1 (B27.29), and ICAM-1 (18E3D).

In an attempt to characterize the MUC1 / ICAM-1 interaction, adhesion of GZHi to 4 h stimulated EAhy926 was studied (section 2.8.). This model preserves the adhesion molecules within the context of their corresponding intact cell membranes and the major adhesive mechanism is largely MUC1—ICAM-1 mediated.

The temperature dependence of the MUC1—ICAM-1 interaction seen in Figure 26, is consistent with an active, dynamic process requiring more than the presence of receptor and ligand on opposing membranes. This requirement is similar to that of the previously described LFA-1—ICAM-1 interaction (98). This adhesion mechanism is susceptible to almost complete inhibition at low temperature (4^{o} C) in the cell—cell adhesion system described above. The reduced adhesion seen at room temperature and the essentially absent adhesion seen at 4^{o} C, may be due to either effects on metabolic energy production or changes in membrane fluidity. These findings are in agreement with observations of cytotoxic T lymphocyte adhesion to target cells which showed LFA-1—ICAM-1 temperature dependence (99).

Similar to the LFA-1—ICAM-1 interaction (45, 98, 100), the MUC1—ICAM-1 interaction in adhesion of GZHi to EAhy926 requires the presence of extracellular divalent cations. Mg^{2+} was the most efficient cation, supporting maximal adhesion at 1mM while Ca^{2+} at similar concentrations supported less than 1/3 of the maximal adhesion. However unlike the LFA-1—ICAM-1 interaction, combination of these cations did not act synergistically to enhance adhesion. In fact the presence of Ca^{2+} offered competition to Mg^{2+} and thus resulted in intermediate levels of adhesion when both cations were present at 1mM (see Figure 27). The competitive inhibition of Mg^{2+} enhanced adhesion by Ca^{2+} suggests there may be only one (or one type) of divalent cation binding site involved in MUC1—ICAM-1 adhesion, which binds either cation with similar avidity but functions optimally when bound with Mg^{2+}.

From these experiments we speculate that MUC1 recognition by ICAM-1 represents an alternate adhesion mechanism active in the late phases or chronic stimulation state of endothelial activation at which point E selectin is no longer present at high levels.

Further support for a MUC1—ICAM-1 interaction can be inferred from studies of interactions between cytotoxic T lymphocytes and MUC1 transfected cells. These interactions are shown to be dependent on ICAM-1 mediated adhesion (93). They are inhibited by the anti-MUC1 core peptide antibody, SM3 (101) and by tumor derived soluble MUC1 (102), which is found in high levels in the circulation of breast cancer patients. This recognition is non - MHC restricted and therefore not based on processed MUC1 peptides. As such, these findings support the hypothesis that adhesion strengthening is at least partially mediated by lymphocyte ICAM-1 binding to the peptide core of MUC1.

The structure of MUC1 is also well suited to an adhesive function by virtue of both its extracellular and cytoplasmic domains. MUC1 has a 69 amino acid cytoplasmic domain which is linked to microtubules of the cytoskeleton (89) as is seen with several other adhesion moieties. The presence of numerous glycosylated tandem repeats allow MUC1 to extend 200-500 nm above the cell surface far beyond the surrounding 10-30 nm glycocalyx (72). This places it in an ideal position to engage in adhesion with those cells possessing the appropriate counter - receptor(s).

Recently, studies have shown that ICAM-1 is recognized by CD43 (leukosialin) (42), another highly glycosylated mucin type glycoprotein, lacking structural resemblance to the β_2 integrin ligands for ICAM-1. In addition, MAdCAM-1, a molecule with mucin and immunoglobulin domains was recently described as a multi-functional adhesion molecule able to interact with both the selectin family of ligands as well as the α_4 integrins. These findings suggest that there is pliancy, and thus built in redundancy in adhesive receptor—ligand recognition so that members of different receptor classes could interact and allow a single molecule to subserve several functions in the absence of the "proper" receptor—ligand pairings. This may be particularly useful to metastasizing cancer cells.

It is thus tempting to speculate that like MAdCAM-1, MUC1 may be a multi-functional adhesion molecule capable of negotiating selectin—carbohydrate binding as well as immunoglobulin superfamily binding.

3.3. The Importance of Secreted MUC1

In advanced breast cancer MUC1 is shed from tumor cells and is thus elevated in the serum where it forms the basis of a recognized marker for metastatic breast

cancer (CA 15-3 or Truquant from Biomira). More recently, elevated serum MUC1 has been directly correlated with an unfavorable prognosis possibly mediating immunosuppression (103).

The endothelium surrounding the tumor embolus in Figure 8b shows moderate positivity for MUC1, suggesting that MUC1 is shed from tumor cells and binds to surrounding endothelium, as endothelial cells do not express MUC1. We have shown that soluble MUC1 can competitively inhibit adhesive interactions of MUC1 positive cells with ICAM-1 (section 2.7.) (Figure 24). Therefore, it is possible that serum MUC1 by occupying ICAM-1 receptors on cytotoxic T lymphocytes may contribute to immune evasion. By occupying peri-tumoral endothelial cell ICAM-1 receptors, serum MUC1 could inhibit adhesive interactions of migrating cells with the endothelium and thus; (1) cause decreased recruitment of inflammatory cells to the tumor site, and (2) facilitate tumor cell escape and metastasis from the primary mass. A similar effect has been proposed for secreted tumor mucin associated carbohydrate blockage of E selectin sites (97).

3.4. Possible ICAM-1 Binding Sites on MUC1

The amount of membrane bound MUC1 is increased in breast cancer. The mucin is also altered with fewer and shorter carbohydrate residues, thereby exposing usually cryptic epitopes associated with the protein core and interior carbohydrates. This cancer associated mucin configuration forms the basis for active specific anti - cancer immunotherapy (87, 104). The MUC1—ICAM-1 interaction in this report was inhibited by the antibody B27.29, an antibody against MUC1's protein core (105). The non-MHC restricted cytotoxic T cell / MUC1 interaction (101) was inhibited by SM3, an antibody which recognizes a

similar sequence within the protein core (77). This suggests that the ICAM-1 binding site on MUC1 lies within the peptide core which is uniquely exposed by the cancer associated underglycosylation of MUC1.

3.5. Conclusions and Implications

It is becoming increasingly evident that mucins have more physiological relevance than simple providers of lubrication. In many adenocarcinomas expression of the MUC1 mucin is up to ten fold higher than that of normal glandular epithelia and is expressed over the entire cell surface, making the molecule easily accessible to interact and / or modulate interactions with other cells.

We have demonstrated tumor cell MUC1, both in cell surface bound and soluble forms, to bind ICAM-1 and have determined this to be an active, Mg^{2+} dependent process. As ICAM-1 is expressed on a variety of cells, its interaction with MUC1 may be very important to breast cancer metastasis. ICAM-1 has been shown to be expressed on stromal fibroblasts surrounding tumor tissue in the majority of breast cancers (106) whereas it is rarely found on fibroblasts in disease free breast stroma. It is quite possible that this expression of ICAM-1 is induced by the adjacent tumor cells and serves as a mechanism by which tumor cells migrate (through interaction with MUC1) across stromal cells on their journey to nearby blood vessels. Upon gaining access to the circulation, tumor cells may home to secondary sites and subsequently negotiate endothelial selectin mediated rolling via recognition of sialylated $Le^{x/a}$ carbohydrates associated with tumor derived MUC1 (95, 96). The mechanism by which tumor cells firmly attach to and migrate through the endothelium may be at least partially explained by our observations of MUC1 mediated adhesion to ICAM-1. Thus,

MUC1 may potentially be capable of mediating the entire tumor cell—endothelial cell adhesion cascade.

Soluble MUC1 may also be important to metastasizing breast cancer cells. By occupying ICAM-1 receptors on cells of the immune system and thereby blocking essential adhesion between immune effector cells and their target tumor cells soluble MUC1 could act as an immunosuppressant. Similarly, soluble MUC1 may contribute to tumor cell survival in vessels proximal to the primary mass, and survival of the primary mass itself by blocking endothelial ICAM-1 promoting tumor cell escape, and inhibiting inflammatory cell infiltration of the tumor.

An important therapeutic feature of the MUC1—ICAM-1 interaction is that MUC1 is virtually restricted in humans to the luminal surface of normal epithelial cells. Thus cytotoxic therapies aimed at MUC1 or disrupting the MUC1—ICAM-1 interaction should have little effect on normal immune function while preventing metastasis.

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